The Structural and Functional Changes of Blood Vessels during Aging

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Dedication

I would like to dedicate this thesis to all those who have supported me throughout this process. To my mom and dad, Diana and Ralph, I thank you for your unwavering support through the peaks and valleys of my research. To my aunt and uncle, Eileen and David, I thank you for your encouragement and belief in my abilities to complete this work. To my sister and brother-in-law, Rachel and Shawn, I thank you for always keeping me grounded and never allowing my work to consume me.

Abstract

The vascular adventitia is recognized as a dynamic mediator of vascular structure and function, yet its role in aging is not understood. The purpose of this thesis was to examine the age-related changes of the vascular adventitia and determine the underlying mediators responsible. Male Sprague-Dawley rats were aged to 15, 30, 50 and 80 weeks before being anesthetised and euthanized by exsanguination. Thoracic aortas, mesenteric and pudental arteries were isolated, formalin fixed, and embedded in paraffin then sectioned at 5μm. Vessels were examined by microscopy and protein expression was determined by indirect immunofluorescence. The thickness of the adventitia increased dramatically with age. Immunofluorescence revealed a robust expression of endothelin system proteins in the adventitia. Additionally, extracellular matrix proteins collagen and fibronectin, and the proliferation marker Ki67 showed strong adventitial origin. The changes observed in the vascular adventitia with aging clearly demonstrate an important role in the process of vascular aging.

Acknowledgments

I would like to thank all of the people without whom this work would not be possible. Thank you to Dr. Johanna Hannan and Mrs. Judy Pang, whose work provided me with the tissues necessary for my analysis. Thank you to Dr. Marina Komolova and Ms. Maria Tina Maio for their technical support. Thank you to Dr. Brian Roy for allowing me to use equipment. Thank you to Drs. Jeff Stuart and Lista Tsiani for providing valuable feedback in the preparation of my thesis. Thank you to Dr. Michael Adams for providing the animals for this study and providing valuable guidance throughout the process of this study. And thank you to Dr. Hui Di Wang for serving as my primary supervisor, providing tremendous ongoing guidance and support, and pushing me to produce high quality work.

Table of Contents

List of Figures and Tables

List of Abbreviations

α-SMA	α-Smooth Muscle Actin
ACh	Acetylcholine
AGE	Advanced Glycation End Product
AMPK	5' Adenosine Monophosphate-Activated Kinase
Ang II	Angiotensin II
ANOVA	Analysis of Variance
AT_1	Angiotensin II Type 1 Receptor
AT_2	Angiotensin II Type 2 Receptor
ATP	Adenosine Triphosphate
bFGF	Basic Fibroblast Growth Factor
BN	Brown Norway
CRP	C-Reactive Protein
CSA	Cross-Sectional Area
cyt p450	Cytochrome p450
DAPI	4',6-Diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
EC	Endothelial Cell
ECE	Endothelin Converting Enzyme
ecSOD	Extracellular Superoxide Dismutase
ECM	Extracellular Matrix
eNOS	Endothelial Nitric Oxide Synthase

ET-1	Endothelin-1
ETA	Endothelin Type A Receptor
ETB	Endothelin Type B Receptor
EtOH	Ethanol
F344	Fisher 344
FB	Fibroblast
H_2O_2	Hydrogen Peroxide
ICAM	Inter-Cellular Adhesion Molecule
IFN-γ	Interferon-gamma
IGF-1	Insulin-like Growth Factor 1
iNOS	Inducible Nitric Oxide Synthase
JNK	c-Jun N-terminal Kinase
L-NAME	ω-Nitro-L-Arginine Methyl Ester
MAPK	Mitogen Activated Protein Kinase
MEK	Mitogen Activated Protein Kinase Kinase
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
NADPH oxidase	Nicotinamide Adenine Dinucleotide Diphoshate Oxidase
NF-κB	Nuclear Factor-kappa-light-chain-enhancer of activated B cells
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NOX	Nicotinamide Adenine Dinucleotide Diphoshate Oxidase

$^{\cdot}O2^{-}$	Superoxide
$^{\cdot}OH$	Hydroxyl Radical
$OONO^{-}$	Peroxynitrite
PBS	Phosphate-Buffered Saline
PDGF	Platelet-Derived Growth Factor
PHOX	Phagocytic Oxidase
PKC	Protein Kinase C
RAAS	Renin-Angiotensin-Aldosterone System
Raf	Raf Proto-Oncogene Serine/Threonine Protein Kinase
Ras	Rat Sarcoma Protein
ROS	Reactive Oxygen Species
SIRT1	Silent Mating Type Information Regulation 2 Homolog 1
SNP	Sodium Nitroprusside
SOD	Superoxide Dismutase
TGF-β	Transforming Growth Factor-beta
TNF-α	Tumor Necrosis Factor-alpha
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cell
vWF	von Willebrand Factor
XO	Xanthine Oxidase

1: Introduction

Although considered for a long time to be merely a passive structural support for the blood vessel wall, the vascular adventitia has now gained attention in cardiovascular research as a potentially critical location for the progression of chronic disease (Rey & Pagano, 2002; Sartore, et al., 2001; Siow & Churchman, 2007). Recent work has revealed the vascular adventitia to be a dynamic tissue capable of participating in disease progression through direct cellular activities and the release of cytokines to act on neighbouring cell types (An, Boyd, Wang, Qiu, & Wang, 2006; Dourron, et al., 2005; Garanich, Mathura, Shi, & Tarbell, 2007). The vascular adventitia thus serves as a potentially untapped wellspring of scientific discovery that could lead to the development of novel treatment strategies for an array of cardiovascular diseases.

Cultured vascular adventitial fibroblasts have been shown to produce and release the potent vasoactive peptide endothelin-1 (ET-1) (An, et al., 2006). ET-1 is commonly implicated in chronic vascular diseases such as hypertension and atherosclerosis. Its effects on the vasculature are widespread, mediating vascular smooth muscle cell (VSMC) contraction (Yanagisawa, et al., 1988), extracellular matrix (ECM) deposition (An, et al., 2007; Rodriguez-Vita, et al., 2005), vascular cell proliferation (Dong, et al., 2005; Milan, et al., 2006), migration (Milan, et al., 2006), and hypertrophy. Conventional wisdom in cardiovascular research has suggested that the production and release of ET-1 is dependent upon the endothelium. However, if these responses are dictated by the vascular adventitia, these results may represent the beginning of a paradigm shift in the orientation of cardiovascular research from an inside-out view to an outside-in view.

Additionally, the vascular adventitia has been implicated in numerous structural

and functional changes in the vasculature, both in health and disease. When exposed to shear stress, adventitial fibroblasts were shown to have enhanced migratory properties, which could contribute to neointimal proliferation (Garanich, et al., 2007). Furthermore, removal of the adventitia severely diminished nitric oxide (NO)-dependent vasodilation, suggesting a paracrine role of the adventitia in vessel relaxation (Beranova, et al., 2005). In addition, adventitial application of a NADPH oxidase inhibitor reduces neointimal proliferation, suggesting the adventitia plays a significant role in atherosclerosis (Chan, et al., 2007). Clearly, the adventitia is an important location when considering chronic vascular diseases.

The process of vascular aging is associated with a tremendous increase in the incidence and prevalence of numerous chronic vascular diseases. The vasculature commonly undergoes structural and functional changes during aging which directly contribute to pathology (Greenwald, 2007; Izzo & Mitchell, 2007). Many of these structural and functional changes mirror changes which occur in the adventitia in response to various injurious stimuli. However, the role that the adventitia plays in vascular aging and contributes to age-related vascular diseases is still unknown. The purpose of this thesis is to elucidate the specific effects of aging on the vascular adventitia, both structurally and functionally. In doing so, we hope to reveal the role of the vascular adventitia in the age-related decline in vascular function.

1.1: The Arterial Tree

The vasculature is a diverse system of conduits which modulate the cyclical pumping of blood produced by the heart. Due to the reciprocal pumping activity of the

heart, the blood vessels must be able to dampen the acute pressure increases achieved at the end of systole in order to produce constant blood flow to the tissues (Greenwald, 2007). Further down the line, the muscular and resistance arteries must dilate and contract to regulate local blood pressure effectively and to maintain the minimal pressure needed to keep blood flowing through the entire systemic circulation (Greenwald, 2007). A disruption of this process in any of these vascular beds results in stiffening of the vessels and an increase in blood pressure.

1.1.1: Aorta & Central Elastic Conduit Arteries

The elastic conduit arteries comprise the thoracic and abdominal aorta, the carotid artery, and the pulmonary arteries. These vessels are the first conduits through which the blood flows after being ejected by the ventricles. As such, these vessels are exposed to the highest temporal blood pressure changes in the entire vascular tree. Due to the very high pressure bolus of blood that each of these vessels receives at end systole, these vessels are highly elastic, and are able to dampen the pressure changes (Greenwald, 2007). The resultant expansion of the aorta during end systole stores kinetic energy and propels the blood throughout the systemic circulation through the duration of diastole (Greenwald, 2007). As such, the proximal aorta may be considered to be the third pumping chamber of the heart (Izzo & Mitchell, 2007).

During aging, the elastic conduit arteries commonly stiffen, losing the elasticity with which they are able to effectively control systemic blood pressure. This stiffening is due to a number of changes, both metabolic and structural. Chief among these changes are the deposition of collagen and breakdown of elastin fibres, calcification of the

vessels, enhanced activity of matrix metalloproteinases (MMPs), accumulation of advanced glycation end-products (AGEs), endothelial cell (EC) dysfunction, and VSMC proliferation and hypertrophy (Greenwald, 2007).

During aging, the aortic wall undergoes substantial stiffening, which may be the result of an overall thickening of the vascular wall. This appears to be achieved through smooth muscle cell hypertrophy (Connat, et al., 2001). Additionally, with age the medial smooth muscle layer of rats becomes increasingly disorganized and irregular, displaying a characteristic folding of the elastic lamellae between smooth muscle cell layers (Connat, et al., 2001). Similarly, carotid artery intima-media thickness increases substantially with age in humans (Tanaka, Dinenno, Monahan, DeSouza, & Seals, 2001). This increase in carotid thickness can be attributed to local elevations in systolic blood pressure (Tanaka, et al., 2001). Furthermore, advancing age is significantly associated with decreased compliance and distensibility of the aorta, but showed no effect in the muscular brachial artery (van der Heijden-Spek, et al., 2000). It would appear that the distending pressure exerted by local elevations in blood pressure commonly observed in aging is sufficient to invoke a hypertrophic response in the large elastic arteries.

A possible mechanism for the thickening of elastic arteries is an activation of inflammatory processes with aging. C-reactive protein (CRP), a marker of cellular inflammation, was shown to be elevated in elderly patients, and correlated well with aortic stiffness (Nakhai-Pour, Grobbee, Bots, Muller, & van der Schouw, 2007). Even when adjusted for traditional risk factors such as smoking and cholesterol levels, CRP significantly predicted increased pulse-wave velocity in these subjects (Nakhai-Pour, et al., 2007). Furthermore, activity of MMP-2 was significantly enhanced in the aortas of

aged subjects (McNulty, Spiers, McGovern, & Feely, 2005). However, the activity of MMP-2 was unchanged with aging in the internal mammary artery (McNulty, et al., 2005). Additionally, in the F344xBN F1 hybrid rat model, a significant increase in inflammation related molecules was observed, including the adhesion molecules inter-cellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), as well as the bone morphogenic proteins osteopontin and bone sialoprotein-1 (S. J. Miller, et al., 2007). In addition to this, the markers of oxidative stress 8-isoprostane and 3-nitrotyrosine were also increased with aging (S. J. Miller, et al., 2007). It would appear that the age-related structural changes observed in the large elastic conduit arteries are mediated at least in part by inflammation and oxidative stress, but these changes are not necessarily observed in smaller muscular arteries further along the arterial tree.

1.1.2: Mesenteric & Peripheral Muscular Arteries

The muscular arteries comprise the blood vessels which are immediately distal to the large elastic conduit arteries in the arterial tree. These arteries are characterized by their greater degree of smooth muscle tone, and an increasing ratio of collagen to elastin content in the ECM (Greenwald, 2007; Izzo & Mitchell, 2007). As a result, these arteries are more sensitive to acute changes in vascular tone, and are often considered to be the most important mediators of essential hypertension (Schiffrin, 2003). Due to their wide distribution around the body and their substantial muscular tone, these arteries have the ability to control peripheral resistance and hypertension (Schiffrin, 2003).

In rat mesenteric arteries, aging induces a significant increase in medial thickness and pulse pressure despite no changes to lumen diameter (Moreau, d'Uscio, & Luscher,

1998). The same arteries exhibited a blunted vasomotor response to Ang II, but no change in sensitivity to either epinephrine or ET-1 (Moreau, et al., 1998). Furthermore, it would appear that the constrictor role of ET-1 in small mesenteric arteries is mediated entirely by the ET_A receptor (Mickley, Gray, & Webb, 1997; Rizzoni, et al., 1997). Given our knowledge of angiotensin II (Ang II) signalling being mediated in part by ET-1 production and release (An, et al., 2006; An, et al., 2007), it is tempting to speculate the changes in Ang II sensitivity with age are reflected in the ability of the vessel to produce ET-1. Interestingly, it has been hypothesized that angiotensin AT_2 receptor activity may switch to more constrictor roles in aging (Widdop, Vinh, Henrion, & Jones, 2008). However, earlier reports noted an increase in aortic ET-1 mRNA production with increasing age (Kumazaki, Fujii, Kobayashi, & Mitsui, 1994; Tokunaga, et al., 1992). Clearly, this demonstrates the variability in the activity of the vasculature at different points in the vascular tree.

Interestingly, the adventitia appears to play a significant role in small artery remodelling with increasing age. The amount of collagen deposition was significantly enhanced with aging in rat mesenteric arteries (Briones, Salaices, & Vila, 2007). This would be in agreement with findings demonstrating increased thickness and stiffness of aged mesenteric arteries in rats (Laurant, Adrian, & Berthelot, 2004). This study also found an overall decrease in systolic and diastolic blood pressure with age, resulting in lower mean arterial pressure. However, the researchers also noted a significant increase in pulse pressure with age, suggesting that the reduced distensibility of the mesenteric arteries was contributing to larger variation in blood pressure (Laurant, et al., 2004). Interestingly, previous work has shown that pulse pressure is correlated with

media/lumen ratio of resistance arteries in elderly patients independent of other common blood pressure measures (James, Watt, Potter, Thurston, & Swales, 1995). It would seem that high pulse pressure, a hallmark of large elastic artery stiffening, plays a role in small artery remodelling.

An important characteristic of small muscular arteries is their ability to control peripheral resistance through the regulation of vascular tone. With increasing age, it was found that the ET-1 mediated vasoconstrictor tone was enhanced in men (Van Guilder, Westby, Greiner, Stauffer, & DeSouza, 2007). This increased vasoconstrictor tone appeared to be mediated greatly by ET_A receptor stimulation, a finding that is confirmed in animal studies (Mickley, et al., 1997; Rizzoni, et al., 1997). Interestingly, prehypertensive renin-angiotensin-aldosterone system (RAAS) blockade results in long-term amelioration of vascular function and structural changes (Baumann, et al., 2007). This blockade also resulted in an enhanced expression of endothelial NO synthase (eNOS) in the vasculature, suggesting a further mechanism for the amelioration of blood pressure augmentation through antihypertensive treatment (Baumann, et al., 2007). Furthermore, the enhanced ET-1 mediated vasoconstrictor tone observed in aging was ameliorated through aerobic exercise training, revealing another mechanism for the long-term augmentation of vascular tone in small arteries (Van Guilder, et al., 2007). It would appear that the long-term elevation in blood pressure in aging mediated by small artery remodelling has numerous causes and similarly may be ameliorated through numerous treatments.

1.1.3: Pudendal & Small Resistance Vessels

The small resistance vessels comprise the arterioles and capillaries that are responsible for gas, nutrient and waste exchange in the cardiovascular system. These vessels have the smallest diameter of any vessel in the body (arbitrarily defined as any vessel less than ~300μm in diameter), and thus may provide the greatest amount of resistance to blood flow (Mitchell, 2008). As with the peripheral muscular arteries, the regulation of blood flow and pressure in the resistance vessels is accomplished through local mediators. As such, the degree of vascular smooth muscle tone in these vessels is paramount in the maintenance of blood flow and pressure (Mitchell, 2008).

As with the vasculature as a whole, the reactivity of the small resistance vessels varies by location (J. M. Muller-Delp, 2006). In aging, the effects of various stimuli differ between the vascular beds of different organ systems (J. M. Muller-Delp, 2006). Additionally, the reactivity of resistance vessels of a single organ system such as the skeletal muscle varies by muscle and fibre type distribution (J. M. Muller-Delp, 2006). For instance, vasodilation to acetylcholine (ACh) is reduced in soleus muscle feed arteries and first order arterioles of aged rats (Woodman, Price, & Laughlin, 2002) however, ACh-induced vasodilation is not affected by aging in gastrocnemius muscle feed arteries and first order arterioles (J. Muller-Delp, et al., 2002).

Similarly, alterations in vasoactive mediator reactivity are common among resistance vessels in aging. The Ang II AT_2 receptors mediate vasoconstriction and reactive oxygen species (ROS) production in aged rats, but produce the opposite effects in young rats (Pinaud, et al., 2007). Additionally, the vasoconstrictive effects of ET-1 in small subcutaneous arteries are mediated entirely by ET_A receptors in aged patients, but

may be mediated by both the ET_A and ET_B receptors in younger patients (Porteri, et al., 2002). Furthermore, intraluminal shear stress may induce eNOS mRNA expression and potentiate ACh-mediated vasodilation in the soleus feed arteries of old rats, but has a much lower effect in young rats (Woodman, Price, & Laughlin, 2005).

It is important to note that although the effects of aging in the vasculature may vary by location in the arterial tree, the changes occurring in one location will ultimately affect another location due to alterations in blood flow and pressure (Mitchell, 2008). For instance, the stiffening of the central elastic conduit arteries may have a direct effect on systolic blood pressure through augmentation of pulse wave velocity. This elevation in blood pressure forces more blood to flow through the distal vasculature which may lead to hypertrophic remodelling of the peripheral muscular arteries and resistance vessels (Mitchell, 2008). This in turn may result in increased peripheral vascular resistance, which may further elevate blood pressure, and lead to further hypertrophic remodelling and stiffening of the central elastic conduit arteries (Mitchell, 2008). So, although there may exist discrete differences in the effects of aging on varying isolated regions of the arterial tree, when combined these individual effects may inevitably lead to the same end result.

1.2: Anatomy of the Arterial Wall

The blood vessels can be described as continuous conduit tissue comprised of three distinct layers, the tunica intima, the tunica media, and the tunica adventitia. Each of these layers plays unique roles in the structural and functional maintenance of the blood vessel wall, both in health and disease. The innermost layer of the blood vessel is

termed the tunica intima, and is normally comprised of a single layer of ECs. This layer provides direct contact with the bloodstream and is critically important for endocrine signalling, gas exchange, and waste removal from the vascular wall (Martini, Bartholomew, & Ober, 2003). The middle layer is separated from the tunica intima by the internal elastic lamina and is termed the tunica media. This layer is the most prominent in the vessel wall, comprises mainly VSMCs, and is chiefly responsible for the maintenance of vascular tone (Martini, et al., 2003). The external elastic lamina separates the tunica media from the outermost layer of the blood vessel, termed the tunica adventitia. This layer is primarily composed of fibroblasts, but may contain numerous other cell types, including macrophages, nerve terminals, and stem cells (Hoshino, Chiba, Nagai, Ishii, & Ochiai, 2008; Rey & Pagano, 2002; Sartore, et al., 2001). Beyond the tunica adventitia is a layer of perivascular fat which generally provides insulation and lubrication for the large arteries, but may also be involved in vascular signalling cascades (Barandier, Montani, & Yang, 2005)

1.2.1: The Vascular Adventitia

For years the vascular adventitia was thought to be simply a passive structural support for the vessel wall, producing ECM and providing a location for the residence of terminal nerve fibres (Gutterman, 1999; Sartore, et al., 2001). Recent work has revealed the adventitia to be a dynamic mediator of vascular structure and function, both in health and disease.

1.2.1.1: Cellular Composition

While the tunica intima and tunica media are mainly composed of ECs and VSMCs, the primary cell type in the adventitia is the fibroblast, which does not express α-smooth muscle actin (α-SMA), myosin, nor desmin under normal conditions. However, under certain pathological conditions (e.g. balloon injury) these fibroblasts are activated and proliferate. These previously α-SMA-negative fibroblasts now express α-SMA and myosin, and transdifferentiate into myofibroblasts. Although they express α-SMA, myofibroblasts do not express markers of highly differentiated VSMCs, such as desmin and smoothin (Ronnov-Jessen & Petersen, 1993; Schmitt-Graff, Desmouliere, & Gabbiani, 1994). The adventitia exhibits few VSMCs, ECs (Sartore, et al., 2001), leukocytes (Capers, et al., 1997), and ganglionic cells (Rey & Pagano, 2002), but may contain stem cells (Hoshino, et al., 2008). Figure 1 displays the arrangement of the vessel wall and outlines the cellular composition of each layer.

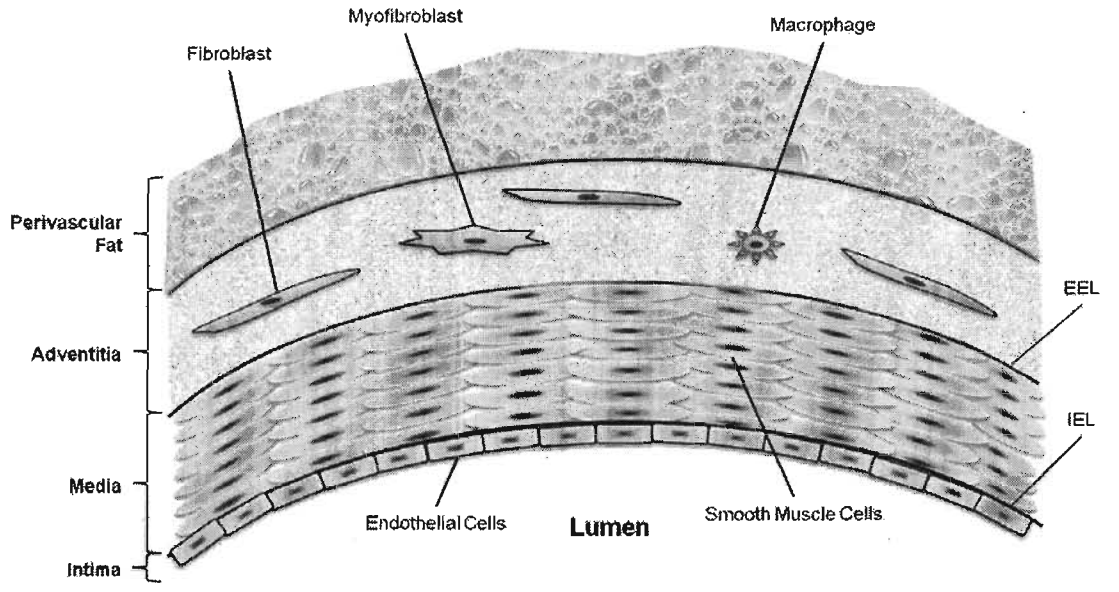

Figure 1. Anatomy and cellular composition of the blood vessel. The tunica intima, composed mainly of endothelial cells, is separated from the tunica media, composed mainly of vascular smooth muscle cells, by the internal elastic lamina (IEL). The external elastic lamina (EEL) separates the tunica media from the tunica adventitia, which is composed of fibroblasts, myofibroblasts, and macrophages, as well as some vascular progenitor cells, and stem cells. Also contained within the adventitia are the vascular terminal nerve fibres. External to the adventitia is a layer of perivascular fat.

1.2.1.2: Vasomotor Responses

Evidence suggests that the vascular adventitia may play an important role in maintaining vascular tone under physiological and pathophysiological states. First, it has been demonstrated that NO derived from adventitial inducible NO synthase (iNOS) can regulate smooth muscle function. Interestingly, results from Kleschyov et al. (1998) demonstrate that the adventitia is a richer source of NO than the media, and that the adventitia-derived NO is able to activate guanylyl cyclase and vasodilatation within the media of the rat aorta (Kleschyov, et al., 1998). Recently, it was reported that the

adventitia is able to produce a 'NO-like' compound that is able to mediate VSMC function in endothelium-denuded rat aortas (Beranova, et al., 2005).

Second, the ability of the adventitia to respond to vasoactive peptides has been discovered recently in human tissue-engineered blood vessels. The results indicate that the engineered adventitia is able to contract after endothelin-1 (ET-1) treatment, a result that was independent of the medial layer and mediated through the ET_A receptor (Laflamme, et al., 2006). Additionally, the tissue-engineered adventitia dilates in response to sodium nitroprusside (SNP) administration (Laflamme, et al., 2006). As a result, the adventitia of the blood vessel may indeed play a significant role in the modulation of vascular tone as it has been demonstrated that both vasoconstriction and vasodilatation can occur in an adventitia-dependent manner.

Third, the adventitial ROS may also play a role in regulating vascular contractile responses. Considerable evidence has indicated that oxidative stress can regulate vascular tone. In Ang II-induced hypertension and spontaneously hypertensive rats, ACh-induced endothelium dependent relaxation is impaired (Park, Touyz, Chen, & Schiffrin, 2002; Rajagopalan, et al., 1996). Interestingly, superoxide dismutase (SOD) markedly increases peak relaxation in response to ACh in vessels from Ang II-treated animals (Rajagopalan, et al., 1996). Consistent with the above findings is the observation by Wang et al. (1999) that adventitial superoxide ($\cdot O_2^-$) is increased dramatically in the Ang II-infused hypertensive model (Wang, et al., 1999). Additionally, it was found that the enhanced $\cdot O_2^-$ production gives rise to spontaneous myogenic contractions (Ghosh, Wang, & McNeill, 2004; Wang, et al., 1999). Strikingly, removing the adventitia from large arteries reduces VSMC contractile responses and enhances endothelium-dependent

relaxation (Gonzalez, Arribas, Molero, & Fernandez-Alfonso, 2001). These findings are consistent with the hypothesis that increased $\cdot O_2^-$ generation, including that by the adventitia, inactivates NO (Karasu, 2000; Wattanapitayakul, Weinstein, Holycross, & Bauer, 2000), and since the diffusion distance for NO is on the order of 30 μm, it is capable of pervading the entire vascular wall (Beckman, 1996). Moreover, $\cdot O_2^-$ can generate hydrogen peroxide (H_2O_2), evoking a loss of endothelial function (Walia, Sormaz, Samson, Lee, & Grover, 2000), thereby inhibiting ACh-induced relaxation and contributing to exaggerated vasoconstrictor responses (Shastri, Gopalakrishnan, Poduri, & Wang, 2002; Wattanapitayakul, et al., 2000). These data strongly support the hypothesis that the vascular adventitia may indeed play a significant role in the modulation of vascular tone. The role of the adventitia in regulating vascular tone through ET-1 expression and NO production is illustrated below in Figure 2.

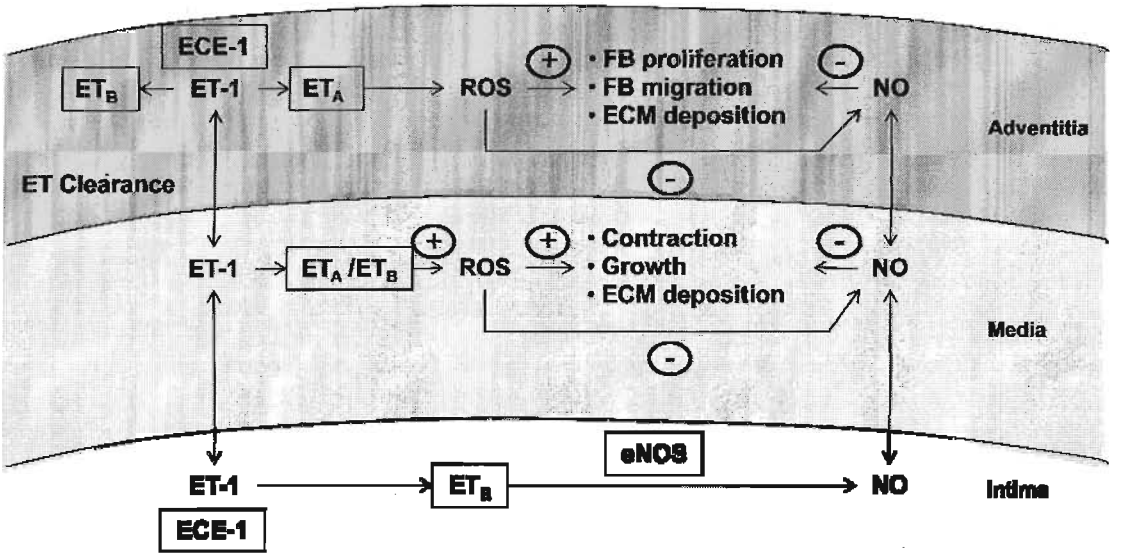

Figure 2. Role of the vascular adventitia in endothelin-1 expression and vasomotor regulation. The endothelin converting enzyme-1 (ECE-1) expressed in the adventitia produces endothelin-1 (ET-1) which may act directly in the adventitia, resulting in extracellular matrix (ECM) deposition, fibroblast (FB) proliferation, and migration, or may diffuse to the media to produce vascular contraction, cell growth, and proliferation. Similarly, the tunica intima is also a rich source of ET-1, but here may act on endothelial ET_B receptors, activating endothelial nitric oxide synthase (eNOS) and the release of nitric oxide (NO), which mediates dilatation in the vascular smooth muscle. ET-1 signalling in the media and adventitia results in reactive oxygen species (ROS) production, which may react with NO, thereby reducing its bioavailability.

1.2.1.3: Role in Vascular Diseases

The vascular adventitia has gained increasing attention as a possible source and cause of vascular diseases, as well as a potential target for therapy. Recent evidence has implicated the vascular adventitial fibroblasts and myofibroblasts in the aetiology and progression of hypertension and atherosclerosis (Chan, et al., 2007; Dourron, et al., 2005; Garanich, et al., 2007; Gonzalez, et al., 2001; Laflamme, et al., 2006; G. Li, Chen, Oparil, Chen, & Thompson, 2000; L. Li, Zhu, Shen, & Gao, 2006; Liu, Ormsby, Oja-Tebbe, &

Pagano, 2004; Shi, O'Brien, et al., 1996). Interestingly, each of these vascular diseases is highly prevalent among aged populations (Hajjar, Kotchen, & Kotchen, 2006; Minamino & Komuro, 2002), lending credence to the possibility of a cardiovascular aging syndrome of adventitial origin. This evidence may be illustrative of the ability of the vascular adventitial fibroblasts to alter the vascular milieu in aging, and contribute to age-dependent stiffening of the arteries. These mechanisms in turn contribute to the progression of hypertension and atherosclerosis.

After intimal injury, the adventitia contributes to the formation of atherosclerotic lesions (Torsney, Hu, & Xu, 2005). In addition to migrating to the site of lesion, the adventitial fibroblasts also release cytokines, recruiting macrophages and monocytes which contribute to further formation of atherosclerotic lesions (Maiellaro & Taylor, 2007). In the context of a hyperlipidemic state, the recruitment of macrophages and monocytes contribute by forming foam cells and oxidizing lipids (Maiellaro & Taylor, 2007).

In addition to the formation and exacerbation of atherosclerotic plaques, the adventitia can also be involved in stabilizing the vessel through vasa vasorum neovascularisation (Ritman & Lerman, 2007). When the atherosclerotic lesion forms, and a plaque results, the vessel lumen becomes greatly reduced. To compensate for this reduction of blood flow and nutrient supply through the vessel lumen, the vessel forms vasa vasora, which are tiny vessels that provide the vessel itself with blood supply (Ritman & Lerman, 2007). Typically vasa vasora are only found in large vessels in which the diffusion of nutrients and waste products through the entire vessel wall is difficult or impossible (Ritman & Lerman, 2007). In atherosclerosis, the new formation of vasa

vasora can occur, providing the vessel with blood flow after the lumen has been occluded. Under hypoxic conditions normally encountered in atherosclerosis, the adventitia is stimulated to release ET-1 (Davie, et al., 2006). ET-1 then acts on the vasa vasora ECs to promote neovascularisation, providing the occluded vessel with blood flow (Davie, et al., 2006). As a side effect, the newly formed vasa vasora serves as a conduit for further macrophage and monocyte migration to the atherosclerotic lesion (Maiellaro & Taylor, 2007). As a result, the atherosclerotic plaque is further exacerbated, and stabilized by vasa vasorum neovascularisation.

The vascular adventitia is well positioned to offer a site for the application of treatments in order to ameliorate the effects of its reactive constituents. Being located on the extreme exterior of the blood vessel, the adventitia allows direct application of pharmaceutical and genetic interventions. Thus far, most of these treatments have revolved around inhibiting the NADPH oxidase system, since this system has been shown to be integral in the regulation of adventitial activation (An, et al., 2007). Chan, et al. (2007) showed that the adventitial application of apocynin (a NADPH oxidase inhibitor) reduces neointima formation and endothelial dysfunction in rabbit carotid arteries. This study, though presenting intriguing results, does not provide definitive evidence of the adventitia acting alone in causing neointima formation and endothelial dysfunction. It does however present evidence of an effective adventitial delivery system that shows a clear improvement in endothelial function compared with more commonly used techniques (Chan, et al., 2007). At the very least, this study provides further *in vivo* evidence illustrating the importance of the adventitia in disease processes. In addition to this study, work with the angiotensin receptor blocker losartan has shown success in

reducing neointimal hyperplasia in porcine arteries (Moon, Molnar, Yau, & Zahradka, 2004). Application of losartan in fibrin glue to the exterior of balloon injured vessels showed a large significant decrease in the amount of ECs growing in the artery (82%), as well as a great reduction in the proliferation of new ECs (97%). This study positively confirms the importance of angiotensin signalling in the adventitia leading to vascular disorders like neointimal hyperplasia.

Further work has examined the introduction of gene therapies to down-regulate the activity of adventitial NADPH oxidase in the vessels. For instance, Liu et al. (2004) showed that the introduction of a viral vector containing the NADPH oxidase inhibitor gp91ds was able to attenuate VSMC hypertrophy in mouse carotid arteries under Ang-II stimulation. As a result of the treatment, the VSMC area remained similar to control conditions, however systolic blood pressure was not returned to control levels (Liu, et al., 2004). The same group also showed that the gp91ds NADPH oxidase inhibitor could also be used to attenuate neointimal formation after balloon injury in rat carotid arteries (Dourron, et al., 2005). Using a very similar dominant-negative $p67^{phox}$ construct, Weaver et al. (2006) were also able to attenuate neointimal hyperplasia in rat carotid arteries. This study also utilized a balloon injury model to induce neointimal formation, and as such may not be directly applicable to chronic disease models of atherosclerosis causing endothelial damage. Nonetheless, the introduction of a natively expressed inhibitor of NADPH oxidase has shown to be effective in reversing the detrimental activities of the adventitia normally found in chronic disease models (Weaver, et al., 2006).

The main drawback of perivascular treatment strategies is the invasive nature of exposing the vessel. Unfortunately there is no effective method for applying a drug or

gene therapy to the exterior of a blood vessel without making a serious incision in the patient. The most readily applicable avenue for the use of perivascular treatments would be in vascular surgery procedures, where the vessel has already been exposed, and the application of the treatment preparation is simply elementary.

1.3: The Role of Endothelin-1 in Vascular Function

1.3.1: ET-1 Biosynthesis

The ET family of peptides consists of three distinct isoforms, which each comprise 21 amino acids. The predominant isoform produced by the vascular endothelium is ET-1 (Yanagisawa, et al., 1988). ET-2 is primarily associated with tumor growth and invasion, whereas the majority of ET-3 is localized to the brain and intestine (Grimshaw, Wilson, & Balkwill, 2002). The biosynthesis of the mature ET-1 peptide begins with the production of a 200 amino acid precursor termed preproET-1. An endopeptidase then cleaves prepro-ET-1 into a 40 amino acid peptide termed bigET-1, which is biologically inactive. Finally, the endothelin converting enzyme-1 (ECE-1) then cleaves bigET-1 into the biologically active mature 21 amino acid peptide, ET-1 (Blais, et al., 2002). The mature ET-1 peptide is approximately 140 times more potent in the vasculature as a vasoconstrictor than bigET-1 (Rubanyi & Polokoff, 1994).

Additionally, it has been shown that MMP-2 may cleave bigET-1 to form a 32 amino acid form of ET-1 (1-32), which displays greater vasoconstrictive effects than that of big-ET-1 (Fernandez-Patron, Radomski, & Davidge, 1999). Furthermore, the big ET-1 precursor peptide may also be cleaved by a chymase, producing a 31 amino acid peptide,

ET-1 (1-31). However, it has been reported that ET-1 (1-31) is most likely bioconverted into the 21 amino acid mature ET-1 since both peptides share similar characteristics (Fecteau, et al., 2005). Once synthesized mature ET-1 peptide is rapidly degraded, displaying a half-life of approximately 1-minute after intravenous injection into the rat (Sirvio, Metsarinne, Saijonmaa, & Fyhrquist, 1990). As such, the activity of ET-1 as a vasoactive mediator is primarily through autocrine and paracrine signalling, since circulating ET-1 is rapidly cleared.

1.3.2: ECE-1

ECE-1 is the main enzyme responsible for the conversion of bigET-1 to ET-1 (Ohnaka, Takayanagi, Nishikawa, Haji, & Nawata, 1993). ECE-1 was first identified as a membrane-bound enzyme (Ahn, Beningo, Olds, & Hupe, 1992), however researchers have since identified at least 5 distinct splice variants, which may be localized intracellularly, or may lack a transmembrane domain (A. R. Hunter & Turner, 2006; Klipper, Levy, Gilboa, Muller, & Meidan, 2006). In human ECs, it was found that the predominant localization of ECE-1 was in small intracellular vesicles called Weibel-Palade bodies (Russell, Skepper, & Davenport, 1998). Similarly, in human and rat VSMCs, the predominant localization of ECE-1 was on the surface of α-SMA filaments (Barnes & Turner, 1999). It would appear from these experiments that the membrane form of ECE-1 is not the most common isoform, thus suggesting an autocrine/paracrine signalling modality of ET-1, rather than dispersion in the bloodstream.

The activity of ECE-1 has been implicated in vascular disease states. Cytokines such as tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) induce the activity of

ECE-1 in VSMCs (Woods, et al., 1999). These same cytokines are commonly involved in inflammatory and proliferatory signalling in atherosclerosis. Furthermore, it was found that ECE-1 protein is abundantly present in atherosclerotic plaques, and may regulate the progression of this disease (Ihling, et al., 2001). Additionally, it was found that ECE-1a and ECE-1c isoforms were localized to both VSMCs and macrophages in atherosclerosis (C. D. Jackson, Barnes, Homer-Vanniasinkam, & Turner, 2006). This would suggest a pivotal role for ECE-1 in the signalling and regulation of atherosclerosis. Potentially, an up-regulation of ECE-1 activity under atherosclerotic conditions may contribute to cell proliferation and the maintenance of vascular tone even in subclinical manifestations of the disease (Ihling, et al., 2001). Interestingly ECE-1 presents another novel target for the treatment of chronic vascular diseases (Grantham, et al., 2000).

More recent work in ECE-1 research has focussed on the specific subcellular localization and regulation of different isoforms and their relation to normal vascular function as well as disease. The ECE-1b, ECE-1c, and ECE-1d isoforms were found to be constitutively phosphorylated *in vivo* when expressed in Chinese hamster ovary cells (MacLeod, Husain, Gage, & Ahn, 2002). Interestingly, it was suggested that a differential phosphorylation of ECE-1 isoforms may regulate which substrate is cleaved by the isoform in each subcellular localization, since each ECE-1 isoform has its own pH optimum, as do its different substrates such as bradykinin, substance P, and bigET-1 (MacLeod, et al., 2002).

Further study has revealed that specific ECE-1 isoforms may be localized to the nuclear compartment (Jafri & Ergul, 2003). Interestingly, under high glucose conditions, the expression pattern of ECE-1 shifted from the plasma membrane to intracellular

locations including the nucleus (Jafri & Ergul, 2003). This would suggest that the intracellular localization of different ECE-1 isoforms may be dependent on the metabolic state of the cell, and regulation of the localization may differ under pathological conditions. Further work has shown that phosphorylation of different isoforms may play a role in subcellular localization (Jafri & Ergul, 2006). Although phosphorylation is not necessary for ECE-1 to be active, this may play a role in regulating the intracellular signalling pathways associated with ET-1 signalling.

1.3.3: ET-1

Endothelin-1 (ET-1) is a potent 21 amino acid vasoactive peptide produced in the vasculature mainly by endothelial cells (Yanagisawa, et al., 1988). First described by Hickey et al (1985) and isolated by Yanagisawa et al (1988), ET-1 has quickly gained attention in cardiovascular research as an important mediator of numerous vascular diseases. Initial reports revealed ET-1 to mediate both vasoconstriction and vasodilatation of vascular smooth muscle, with the action depending on the presence of an intact endothelium (Hirata, et al., 1993; Sumner, Cannon, Mundin, White, & Watts, 1992). Subsequent work has shown that ET-1 is the most potent vasoconstrictive peptide known, and is essential for the control of vascular tone in the muscular arteries.

Although ET-1 is mainly synthesized by ECs under physiological conditions, it can be produced by non-ECs. ET-1, Ang II, thrombin, and other growth factors can induce the expression of preproET-1 mRNA in cultured VSMCs (Sugo, et al., 2001; Tchekneva, Quertermous, Christman, Lawrence, & Meyrick, 1998). ET-1 can also be released from cardiac myocytes (L. L. Yang, et al., 2004) and cardiac myofibroblasts

(Ammarguellat, Larouche, & Schiffrin, 2001; C. M. Cheng, et al., 2003; T. H. Cheng, et al., 2003; Gray, Long, Kalinyak, Li, & Karliner, 1998; Sakai, et al., 1996), where it appears to play a role in both hypertension and post-myocardial infarction (Ammarguellat, et al., 2001; Sakai, et al., 1996). Moreover, it has also been reported that ET-1 released from cardiac fibroblasts mediates cardiac hypertrophy via the ET_A-receptor (Gray, et al., 1998).

Recently, our lab has shown that Ang II stimulation evokes expression of ET-1 in adventitial fibroblasts (An, et al., 2006). The nucleotide sequence of the adventitial preproET-1 cDNA was identical to that of the endothelial preproET-1 cDNA. Importantly, these results demonstrate a potential function of vascular adventitial fibroblasts, since released ET-1 can modulate Ang II-induced ECM generation. Furthermore, the Ang II-stimulated type I procollagen-α-1 mRNA expression and protein synthesis are inhibited by an ET_A-receptor inhibitor, suggesting a potentially meaningful role for this interaction at the functional level.

Oxidative stress may also contribute to ET-1 release. Free radical-generated F2-isoprostane released during oxidative injury results in the release of ET-1 in bovine aortic ECs (Yura, et al., 1999). Additionally, H_2O_2 increases ET-1 expression in human mesangial cells (Hughes, Stricklett, Padilla, & Kohan, 1996), coronary VSMCs (Kaehler, et al., 2002; Kahler, et al., 2001), aortic VSMCs (Ruef, Moser, Kubler, & Bode, 2001), and umbilical vein ECs (Kaehler, et al., 2002; Kahler, et al., 2000). Consistent with the above findings, expression of ET-1 in adventitial fibroblasts is also mediated by $\cdot O_2^-$ (An, et al., 2007). First, $\cdot O_2^-$ generation in Ang II-treated cells increased concomitantly with that of ET-1 synthesis. Second, pharmacological scavengers of $\cdot O_2^-$ or overexpression of

SOD1 have been shown to decrease Ang II-induced ET-1 release. Third, the Ang II-induced ET-1 expression and $\cdot O_2^-$ levels were decreased in cells isolated from $gp91^{phox}$-null mice, suggesting that $gp91^{phox}$ and its derived $\cdot O_2^-$ plays an important role in mediating ET-1 release from adventitial fibroblasts. Nevertheless, this data also suggests that there are mechanisms other than $gp91^{phox}$-containing NADPH oxidase mediating the effect.

The mechanisms of oxidative stress regulating ET-1 production are still unclear. Kahler et al. (2000) reported that oxidative stress increases ET-1 release by activation of a promoter for the ET-1 gene. Oxidative stress could activate nuclear factor-kappa B (NF-κB) (Geng, Rong, & Lau, 1997), which may stimulate preproET-1 gene expression (Bierhaus, et al., 1997). Oxidative stress stimulates the generation of transforming growth factor-β (TGF-β) in glomerular cells (Montero, et al., 2000), which could markedly enhance ET-1 expression in both rat VSMCs and ECs (Sugo, et al., 2001). Both ROS and the Ras-Raf-ERK pathway are required for either Ang II-induced or ET-1-induced ET-1 gene expression in rat cardiac fibroblasts (C. M. Cheng, et al., 2003) . Antioxidants suppress ET-1-induced ET-1 gene expression (C. M. Cheng, et al., 2003), which is consistent with the data obtained in the present study. Inhibition of ERK prevents the transcription of the ET-1 gene (C. M. Cheng, et al., 2003). Likewise, dominant-negative mutants of Ras, Raf, and MEK1 also decrease ET-1 transcription (Cheng, et al., 2001). Meanwhile, the effect of NO on ET-1 expression was reported controversially. Previously, it was found that inhibition of NO can increase ET-1 production (Hinson, Kapas, & Cameron, 1996); however Kahler et al. (2000) reported that the level of NOS III mRNA remained unchanged under oxidative stress, and L-NAME did not

significantly alter ET-1 promoter activity.

Consistent with the findings in ET-1 expression, Ang II-induced procollagen-α-I expression was inhibited by $\cdot O_2^-$ scavengers and NADPH oxidase inhibitors (An, et al., 2007). Moreover, Ang II-induced procollagen-α-I levels were decreased in cells overexpressing SOD1 (An, et al., 2007). Our data agree with the findings reported by other investigators that $\cdot O_2^-$ serves to regulate collagen metabolism in cardiac fibroblasts through the activation of MMPs (Siwik, Pagano, & Colucci, 2001). Hence, Ang II-induced oxidative stress regulates ET-1 release, which in turn mediates collagen synthesis in adventitial fibroblasts. These findings have important implications for disease states associated with remodelling of the vasculature. Rocnik et al. (1998) reported that vascular injury can induce a sequence of events in the adventitia that includes the deposition of newly synthesized collagens, which in turn is a key factor for VSMC migration. Moreover, collagen accumulation contributes to vascular stiffness and changes in vascular compliance. Therefore, these findings reveal the mechanism that contributes to ET-1 release and collagen deposition in the overall remodelling of the arterial wall in various pathological conditions and vascular aging.

1.3.4: ET Receptors

1.3.4.1: ET_A

The ET_A receptor was initially shown to mediate vasoconstriction in the vasculature, both in endothelium-intact and endothelium-denuded arteries (Sumner, et al., 1992; Taddei & Vanhoutte, 1993). The ET_A receptor is expressed in both ECs and

VSMCs, and may mediate vasoconstriction by acting at each of these sites (Taddei & Vanhoutte, 1993). Our lab has also recently shown that the ET_A receptor is expressed in cultured mouse aortic adventitial fibroblasts (An, et al., 2006), a finding which has been confirmed in a tissue-engineered blood vessel model (Laflamme, et al., 2006). In this tissue-engineered model, it was also shown that the ET_A receptor mediated contractile responses in the adventitia-only constructs (Laflamme, et al., 2006).

Beyond the well documented vasoactive properties of the ET_A receptor in the vasculature, the ET_A receptor may also mediate numerous growth and proliferatory responses. In the long-term, ET_A receptor antagonism reduces blood pressure, and attenuates vascular remodelling in hypertensive rats (Park & Schiffrin, 2001). In the heart, chronic ET-1 signalling through the ET_A receptor mediates cardiac fibrosis through collagen deposition (Ammarguellat, et al., 2001). Similarly, we have shown attenuated collagen production by adventitial fibroblasts after ET_A receptor blockade (An, et al., 2006). Furthermore, we and others have demonstrated an activation of ROS-producing pathways, especially the NADPH oxidase pathway, with ET-1 signalling through the ET_A receptor (An, et al., 2007; K. J. Dammanahalli & Z. Sun, 2008; Laplante, Wu, Moreau, & de Champlain, 2005; Zeng, Zhou, Yao, O'Rourke, & Sun, 2008). These pathways commonly manifest in disease states, and often regulate growth and proliferation.

The ET_A receptor is also strongly implicated in disease processes such as atherosclerosis and hypertension. After balloon injury of the rat carotid artery, it was shown that the ET_A receptor is up-regulated in the intimal and medial layers, suggesting a role for the ET_A receptor in injury-induced cell proliferation (Viswanathan, De Oliveira, Johren, & Saavedra, 1997). Furthermore, ET_A receptor blockade resulted in a modulation

of EC adhesion molecule content, thus altering endothelial cell-leukocyte interactions, a hallmark of vascular inflammatory diseases (Callera, et al., 2004). Additionally, ET_A signalling resulted in decreased expression of eNOS, which further exacerbated a model of pulmonary hypertension (Wedgwood & Black, 2005). Thus, in experimental models of cardiovascular disease, ET_A receptor blockade is generally effective in ameliorating the syndrome.

1.3.4.2: ET_B

The ET_B receptor first gained attention for mediating vasodilatory responses when stimulated on ECs (Hirata, et al., 1993), but may mediate vasoconstrictive responses when stimulated on VSMCs (Sumner, et al., 1992). The ET_B receptor has since been classified as two distinct receptors which arise from alternative splicing of the ET_B receptor gene. By convention, the ET_B receptor mediating vasodilatation is termed ET_{B1}, while the ET_B receptor mediating vasoconstriction is termed ET_{B2} (Shyamala, Moulthrop, Stratton-Thomas, & Tekamp-Olson, 1994). The ET_B receptor displays no preference for either ET-1, ET-2 or ET-3, although the ET_A receptor shows significantly higher affinity for ET-1 and ET-2 over ET-3 (Sakurai, Yanagisawa, & Masaki, 1992; Sakurai, et al., 1990). Generally, the ET_B receptor has gained notoriety for its ability to mediate beneficial effects of ET-1, rather than the detrimental effects that the ET_A receptor is known to mediate.

In the pulmonary vasculature, the ET_B receptor has been shown to mediate clearance of the ET-1 peptide from the circulation (Dupuis, Goresky, & Fournier, 1996; Fukuroda, et al., 1994). It was speculated and later confirmed that the clearance of ET-1

mediated by ET_B receptors is dependent on the binding of ET-1 to the ET_B receptor and an internalization and degradation of the ET-1/ET_B complex (Dupuis, et al., 1996; Fukuroda, et al., 1994; Sanchez, et al., 2002). It has since been demonstrated that the ET_B receptor regulates the production of ET-1 peptide and mRNA synthesis in vascular cells (Farhat, et al., 2008; Ozaki, et al., 1995; Sanchez, et al., 2002). Thus the ET_B receptor may reduce the detrimental effects of ET-1 in the vasculature partly by clearing it from the circulation and down-regulating its production, thus serving as a negative feedback on ET-1.

Recent work has shown that the ET_B receptor may mediate anti-proliferative functions in the vasculature. Dammanahalli and Sun (2008) showed that the ET_B receptor mediates the inhibition of NADPH oxidase in human abdominal aortic ECs. Similarly the ET_B receptor agonist Sarafotoxin 6c reduces infarct size in the ischemic/reperfused myocardium of anaesthetized rats (Crockett, Gray, Kane, & Wainwright, 2004). Furthermore, ET_B receptors in the endothelium mediate the production of NO, which besides its well documented vasodilatory actions, may mediate anti-proliferatory responses in the vasculature (Hirata, et al., 1993). Additionally, ET_B receptor stimulation results in suppression of EC apoptosis (Shichiri, Marumo, & Hirata, 1998). Thus the role of the ET_B receptor in the mediation of ET-1 signalling in the vasculature is largely beneficial, whereas the role of the ET_A receptor is not.

1.4: Vascular Aging

The process of aging has long been considered a relative mystery in the scientific community. The exact reasons and mechanisms by which we age have never been well

understood. Numerous theories as to the origins of the aged phenotype have arisen over the years. For the purposes of this review, only the biological theories of aging will be addressed, since the social theories of aging are not of concern here.

1.4.1: Chronological Aging

The simplest concept of aging is the accumulation of changes to an organism over time which is commonly referred to as chronological aging. This model of aging also serves as the most straightforward to test in an experimental approach since one need not assay biomarkers of aging to confirm the animal's age. This model of aging also takes into account the impact of time on the aging phenotype. Numerous specific theories of aging propose that the process of aging is a direct result of the accumulation of damage, waste, or misrepair (Kulminski, et al., 2007; R. A. Miller, 2009). Each of these theories portrays aging as an inevitable, degenerative process; however they do not explain individual differences in aged subjects.

1.4.2: Biological Aging

The process of aging is often explained by biological changes that occur over time. Such a theory of aging is commonly referred to as the biological theory of aging and reflects the apparent age of an organism, rather than its actual measurable age. Often the concept of biological age is used to explain differences in health and disease from similar aged patients, and increasingly is used clinically in determining the efficacy of planned treatment options (S. H. Jackson, Weale, & Weale, 2003). In order to accurately assess biological age, numerous biomarkers of biological aging have been devised,

including oxidative stress (Ungvari, et al., 2008), telomere length (Slijepcevic, 2008), AGEs (Safciuc, et al., 2007), and alterations in bone mineral density.

Underlying mechanisms proposed for biological aging are wide-ranging and diverse. The most common theories involve mitochondrial free radical generation (Gruber, Schaffer, & Halliwell, 2008) and accumulative oxidative stress resulting in DNA and protein damage (Ungvari, et al., 2008). Consistent with these theories is the notion of damage and mis-repair, where a cell manifests aging in terms of incorrect repairing of accumulative damage over time. In either case, the resulting phenotype is that which is common among the aged population, a general deterioration of structural integrity and biological function.

The mitochondrial free radical theory of aging proposes that damage caused by free radicals generated by mitochondria accumulates over time, leading to the common aged phenotype (Gruber, et al., 2008). The main targets of mitochondrial free radicals are DNA, both mitochondrial and nuclear. The mitochondrial DNA damage usually leads to further mitochondrial dysfunction and an increase in the production of free radicals, resulting in a vicious cycle of mitochondrial damage and dysfunction (Gruber, et al., 2008). The nuclear DNA damage severely limits the cell's ability to replicate and function normally. However, the *Mclk1*$^{+/-}$ mouse variant displays significantly increased mitochondrial oxidative stress and dysfunction, but paradoxically lives significantly longer than their wild-type counterparts (Lapointe & Hekimi, 2008). Clearly, the mitochondrial free radical theory of aging is one that requires reformulating, and may no longer be valid in its current state.

Another common theory explaining biological age is telomere length. Telomeres

are repeated sequences of DNA at the ends of linear eukaryotic chromosomes which shorten with every replication due to the limitations of normal DNA replication enzymes (Aubert & Lansdorp, 2008; Lansdorp, 2009). As the organism ages, telomeres shorten until they finally erode away, exposing the coding sequences of DNA. At this point, in the absence of activated telomerase, the DNA damage can be severe enough as to prevent normal replication and the cell dies as a result (Aubert & Lansdorp, 2008; Lansdorp, 2009). Thus the measurable length of a telomere can accurately portray the effective age of an organism. Shortened telomere length has been shown to be associated with hypertension (Z. Yang, et al., 2009), coronary artery disease (Ogami, et al., 2004), myocardial infarction (Zee, Michaud, Germer, & Ridker, 2009), EC dysfunction and atherosclerosis (Minamino & Komuro, 2002). Similarly, long telomere length is associated with relatively good health in aged populations (Terry, Nolan, Andersen, Perls, & Cawthon, 2008).

1.4.3: Structural and Functional Changes during Aging

Vascular aging is a multi-faceted alteration of the vasculature with increasing age. Many common vascular diseases such as hypertension and atherosclerosis can be attributed, at least in part, to age-related changes in the structure and function of the vasculature (Greenwald, 2007; Izzo & Mitchell, 2007). The most common changes seen in vascular aging include vascular cell hypertrophy, cell proliferation and apoptosis, ECM deposition, and oxidative stress. Each of these factors contributes alone and in combination to create an aged phenotype where common chronic vascular diseases thrive. Each of these factors has been shown to be directly influenced by the vascular

adventitia, although the specific role of the adventitia in aging is yet to be determined.

1.4.3.1: Vascular Hypertrophy

Aging has long been associated with structural changes in the vasculature. As the vessels age, the elastic compliance of the vessels decreases in a process called arteriosclerosis (Izzo & Mitchell, 2007). This decrease in compliance is directly influenced by an increase in cell mass of the vessel wall and overall thickening of the vessel (Izzo & Mitchell, 2007). The resultant thickened and stiffened vessel loses its ability to dampen transient blood pressure peaks caused when the heart ejects blood during systole. As a result, blood pressure increases substantially, leading to hypertension, which is further associated with additional cardiovascular outcomes, such as myocardial infarction and stroke.

Briones and colleagues found that aged rat mesenteric arteries demonstrate an overall increase in vessel wall thickness, which could be explained by a correlative increase in the thickness of the medial smooth muscle layer (Briones, et al., 2007). The researchers did not observe a significant increase in the thickness of the adventitial layer, however did observe an increase in the number of cells residing in the adventitial layer (Briones, et al., 2007). The hypertrophic changes observed in this study were also consistent with previous findings in aged rat mesenteric arteries (Adrian, Laurant, & Berthelot, 2004; Moreau, et al., 1998).

Furthermore, it was found that carotid artery thickness in aging could be wholly explained by increases in systolic blood pressure (Tanaka, et al., 2001). However, this study did not quantify changes in the adventitia and only determined carotid intima-

media thickness in assessing vessel wall hypertrophy. Although this study may more accurately demonstrate the age-dependent changes in the large elastic conduit arteries, it provides no assessment of the adventitia. On the other hand, work done by the same group with muscular femoral arteries showed that age-induced thickening is related to enhanced sympathetic nervous activity (Dinenno, Jones, Seals, & Tanaka, 2000). Thus, the effects of age in inducing hypertrophic changes in the vasculature may relate to the action of local mediators on specific locations in the arterial tree, since the elastic carotid artery is influenced by factors which do not affect the muscular femoral artery.

It is well established that many chronic vascular diseases result in pronounced vascular cell hypertrophy. Exposure to pressor doses of Ang II for two weeks results in salt-sensitive hypertension in rats (Lombardi, et al., 1999). This hypertension was shown to be mediated by structural changes in the renal and cardiovascular systems (Lombardi, et al., 1999). Further study revealed that chronic Ang II-induced vascular hypertrophy is mediated by activation of NADPH oxidase and $\cdot O_2^-$ production (Wang, Johns, Xu, & Cohen, 2002; Wang, et al., 2001). Meanwhile, it was found that Ang II signalling caused hypertrophy of cardiac myocytes and that this hypertrophy was mediated by TGF-β and ET-1 release by neighbouring cardiac fibroblasts (Gray, et al., 1998). Evidence from our own lab has shown that Ang II signalling in cultured aortic adventitial fibroblasts can stimulate ET-1 production and release through similar mechanisms (An, et al., 2006; An, et al., 2007). Thus, Ang II and ET-1 signalling in the vasculature may play a significant role in the age-related changes in vascular structure and function. Furthermore, the adventitia may play a role in regulating Ang II and ET-1 signalling through its capacity for paracrine signalling in the vessel wall.

1.4.3.2: Cell Proliferation and Apoptosis

Aging is commonly associated with structural changes of the vessel wall, leading to many age-related cardiovascular diseases. One such change in the vasculature with aging is cell proliferation and apoptosis. Briones et al found that aging resulted in a significant increase in the number of adventitial cells, but resulted in no change in VSMCs (Briones, et al., 2007). Interestingly, another study found that senescent human fibroblasts express and release vascular endothelial growth factor (VEGF) to a much greater degree than pre-senescent fibroblasts (Coppe, Kauser, Campisi, & Beausejour, 2006). This enhanced release of VEGF contributed to increased tumorigenesis and neovascularisation when injected into mice with tumorigenic mouse epithelial cells (Coppe, et al., 2006). Similarly, hypoxia induces the emergence of pulmonary artery adventitial fibroblasts which lack the anti-replication signal arising from protein kinase C-ζ (PKCζ) (Das, Burns, Wilson, Zawada, & Stenmark, 2008). Interestingly, antagonizing the effects of TNF-α, a regulator of apoptotic cell death, in aged rats decreased the expression of inflammatory markers and exhibited vasculoprotective effects (Csiszar, et al., 2007). Thus, cell regulators of proliferation and apoptosis appear to play a role in vascular aging phenotypes.

As with vascular hypertrophy, ET-1 signalling stimulates proliferation of VSMCs (Wedgwood, Dettman, & Black, 2001), ECs (Dong, et al., 2005), and adventitial fibroblasts (Davie, et al., 2006). Similar proliferatory responses are observed in vascular cells upon exposure to extracellular adenosine triphosphate (ATP) (Gerasimovskaya, et al., 2002), hypoxia (Das, et al., 2001), F2-isoprostane (Yura, et al., 1999), and platelet-derived growth factor (PDGF) (Zhan, et al., 2003). Intriguingly, resveratrol, a red-wine

polyphenol, can reverse some aspects of vascular aging. Resveratrol can inhibit Ang II-induced ET-1 gene expression and subsequent cell proliferation in aortic VSMCs (Chao, et al., 2005). Additionally, resveratrol can protect ECs from oxidative stress induced apoptosis (Ungvari, et al., 2007).

1.4.3.3: Extracellular Matrix Deposition

Aging is commonly associated with stiffening of the blood vessels, particularly the central elastic conduit arteries, which is often due to an excess or dysregulated production of ECM proteins (Greenwald, 2007). As an animal ages, the ratio of elastin to collagen in the vessel wall drops, the vessel wall stiffens, and is unable to effectively dampen the acute blood pressure increases experienced during end systole (Greenwald, 2007). As a result, the blood pressure rises substantially, and the blood vessels compensate by growing thicker. This thickening is often accompanied by an enhanced secretion of ECM proteins, primarily collagen, which further stiffens the vessel wall (Greenwald, 2007). The resultant remodelled vessel wall may now provide additional scaffolding for the migration of fibroblasts (Greiling & Clark, 1997) or VSMCs (Rocnik, et al., 1998), which are hallmarks of chronic vascular pathologies such as atherosclerosis.

The vascular adventitia is chiefly responsible for producing copious ECM proteins, particularly collagen and elastin, which maintain structural support of the vessel wall (Rey & Pagano, 2002). Although physiological levels of ECM are required for normal vascular function, the accumulation of interstitial collagen is associated with vascular disease (Intengan & Schiffrin, 2001; Park & Schiffrin, 2001). Excessive deposition of ECM proteins, especially collagen, due to pathological conditions such as

systemic hypertension, occurs throughout the vessel with initial build-up taking place in the adventitial region of arterioles (Nicoletti & Michel, 1999). Increased production of collagen has been demonstrated in adventitial fibroblasts due to stimulation with several mitogens, including Ang II (An, et al., 2006) and ET-1 (An, et al., 2007). The increased synthesis of procollagen, the precursor of collagen has been observed in cardiac fibroblasts by several other substances and forces, including mechanical load (Carver, Nagpal, Nachtigal, Borg, & Terracio, 1991), PDGF, basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1) (Bishop & Lindahl, 1999). TGF-β also serves to up-regulate procollagen production in both cardiac fibroblasts (Bishop & Lindahl, 1999) and interstitial fibroblasts of the pulmonary vascular system (Lindahl, et al., 2002). Additionally, stimulation with TGF-β, PDGF, or bFGF leads to a decrease in collagen degradation (Bishop & Lindahl, 1999).

The production of another ECM protein, fibronectin, is also proving to be of significance as it is involved in many complex cellular functions including tissue repair (Briggs, 2005), embryogenesis (Wartiovaara, Leivo, Virtanen, Vaheri, & Graham, 1978), and blood clotting (Holland, Harlos, & Blake, 1987). Indeed, fibronectin produced by adventitial fibroblasts is essential for the conversion of fibroblasts to activated myofibroblasts (Gabbiani, 2003). The role of fibronectin in cell migration has been demonstrated to occur following injury (Greiling & Clark, 1997), with insoluble fibronectins bringing together anchoring cells with collagen or proteoglycan substrates (A. W. Hahn, Regenass, Kern, Buhler, & Resink, 1993).

1.4.3.4: Oxidative Stress

A state of oxidative stress occurs when the balance of oxidant molecules outweighs that of the antioxidants. As a result ROS can overrun the cell, causing severe damage to cellular components. Although ROS may have important signalling purposes in vascular cells, an overproduction of ROS may commonly occur in numerous vascular diseases including atherosclerosis and hypertension. The predominant ROS produced by vascular cells include $\cdot O_2^-$, H_2O_2, hydroxyl radical ($\cdot OH$), and peroxynitrite ($OONO^-$). Generally, $\cdot O_2^-$ is the first ROS produced by vascular cells. All vascular cells can generate $\cdot O_2^-$ (Griendling, Minieri, Ollerenshaw, & Alexander, 1994; Mohazzab, Kaminski, & Wolin, 1994; Pagano, et al., 1995), with different cell types containing various $\cdot O_2^-$ generating systems. Xanthine oxidase (White, et al., 1996), NADH oxidase (Munzel, Hink, Heitzer, & Meinertz, 1999), and NOS (Munzel, et al., 2000) are present in ECs, while NADPH oxidase is present in VSMCs (Griendling, et al., 1994; Ushio-Fukai, Zafari, Fukui, Ishizaka, & Griendling, 1996), ECs (Gorlach, et al., 2001) and adventitial fibroblasts (Pagano, Chanock, Siwik, Colucci, & Clark, 1998). Additionally, vascular cytochrome P450 (cyt P450) can also generate $\cdot O_2^-$ and is expressed in virtually all vascular cell types (Fleming & Busse, 2001; A. L. Hunter, Bai, Laher, & Granville, 2005). Figure 3 depicts some of the key ROS generating systems and pathways present in the vasculature.

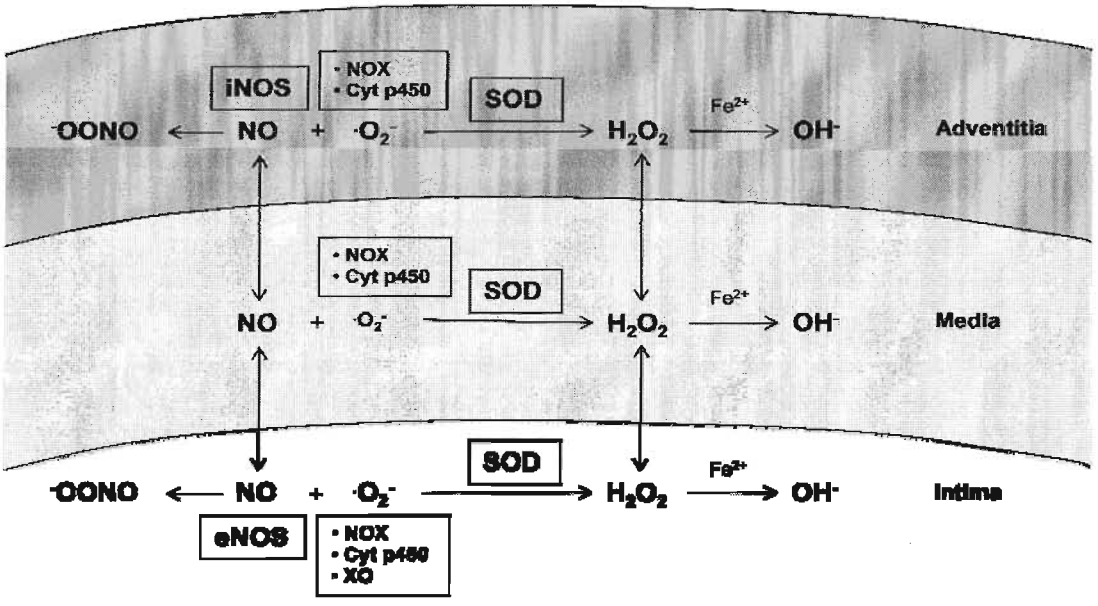

Figure 3. Key ROS generating systems and pathways present in the vasculature. The vascular adventitia is a potent source of superoxide anion ($\cdot O_2^-$), which may combine with nitric oxide (NO) diffused from the endothelium or generated locally by inducible nitric oxide synthase (iNOS) to produce the highly reactive and destructive peroxynitrite ($OONO^-$). Additionally the tunica media and tunica intima are also able to generate $\cdot O_2^-$. The vascular $\cdot O_2^-$ may be dismutated into hydrogen peroxide (H_2O_2), which may serve as a signalling molecule or as a destructive reactive oxygen species. The H_2O_2 may also be decomposed to form hydroxyl radical ($^{\cdot}OH$), another damaging reactive oxygen species. Abbreviations used: NOX, NADPH oxidase; cyt p450, cytochrome p450; XO, xanthine oxidase; eNOS, endothelial nitric oxide synthase.

An important application of oxidative stress in the vasculature is the cumulative effect of ROS in aging and chronic disease processes. Autopsied patients who had suffered from pulmonary hypertension were shown to have greatly thickened adventitial layers, due to increased fibroblast proliferation and collagen deposition (Haurani & Pagano, 2007). Evidence from studies examining hypoxia (Das, et al., 2001), acute vascular injury (G. Li, et al., 2000), shear stress (Garanich, et al., 2007) and ROS

generation (Shen, et al., 2006) have shown the capabilities of the adventitia in responding to a wide variety of stresses. In each case, the adventitial fibroblasts proliferate, differentiate, and migrate, resulting in thickened, stiffened vessels. As a consequence, the vessel is not able to dilate as effectively and efficiently, resulting in a common chronic state of high blood pressure. The high blood pressure experienced can further exacerbate the condition, contributing more shear stress and acute vessel injury, resulting in a vicious cycle of further thickening and stiffening.

Vascular oxidative stress has been reported to mediate vascular hypertrophy. It has been reported that H_2O_2 generated from NADPH oxidase-derived $\cdot O_2^-$ plays a critical role in Ang II-induced hypertrophy of cultured VSMCs by activating growth related signalling pathways and growth factors (Ushio-Fukai, Alexander, Akers, & Griendling, 1998; Ushio-Fukai, et al., 1999). Earlier studies suggested a paracrine effect of ROS on medial hypertrophy (Zafari, et al., 1998). Subsequently, it has been reported that Ang II-induced NADPH oxidase-derived ROS in the adventitia and intima of the aorta is concomitant with medial hypertrophy (Wang, et al., 1999). Overexpression of human catalase in VSMCs decreased Ang II-induced hypertrophy *in vivo* (Zafari, et al., 1998), and adventitia-specific expression of a peptide that inhibits the ability of $gp91^{phox}$ and $p47^{phox}$ to interact, decreases NADPH oxidase activity in the adventitia, and reduces both medial ROS levels and medial hypertrophy (Liu, et al., 2004). These data strongly support the paracrine effect of adventitial NADPH oxidase-derived ROS, primarily H_2O_2, on vascular hypertrophy.

Adventitial fibroblasts are involved in arterial repair (G. Li, et al., 2000; Shi, Pieniek, et al., 1996) and serve as one of the major sources of vascular ROS production

(Ardanaz & Pagano, 2006; Pagano, et al., 1997; Wang, et al., 1999; Wang, et al., 1998). Inhibition of adventitial ROS production has been demonstrated to decrease neointimal proliferation (Dourron, et al., 2005), hyperplasia (Weaver, et al., 2006), and VSMC hypertrophy (Liu, et al., 2004). An intriguing study by Haurani et al. (2008) demonstrated that a reduction in NADPH oxidase-derived ROS also reduces adventitial fibroblast migration *in vitro*. Furthermore, adventitial overexpression of extracellular superoxide dismutase (ecSOD) significantly reduces neointimal formation in a cuff-injured artery model (Ozumi, et al., 2005). These results would strongly suggest that adventitial-derived ROS, generated by NADPH oxidase, are a critical mediator of vascular wall remodelling and repair following injury.

Oxidative stress and reactive oxygen species play a major role in the differentiation, proliferation and migration of adventitial fibroblasts. Ang II has been shown to mediate adventitial fibroblast differentiation to myofibroblasts via NADPH-oxidase ROS through activation of p38-MAP kinase and JNK pathways (Shen, et al., 2006). In this response, Ang II mediates an increase in ROS generation by NADPH oxidase. The subsequent increase in H_2O_2 appears to be important in activating the p38-MAPK and JNK pathways, which mediate gene expression of α-SMA, which serves to signal the cell to differentiate to a myofibroblast (Shen, et al., 2006).

1.4.4: Animal Models used in the Study of Aging

When studying aging of the cardiovascular system, it is useful to devise a comparable animal model that can be used to mimic the age-related changes observed in humans. The most commonly used animal aging model in cardiovascular research

involves common laboratory rat strains such as Fisher 344 (F344) or Brown Norway (BN) (Kajstura, et al., 1996; Pacher, et al., 2004). The F344 rat is an inbred strain selectively bred by the National Institute of Aging to have smaller body mass, longer lifespan and lower spontaneous tumour rate than most other rat strains, making them somewhat more comparable to humans in aging studies (Pacher, et al., 2004). These rats generally have a maximum lifespan of approximately 3.0 years (36 months). The generally accepted standard when using these rats is to use 3-6 month old rats for young subjects and 24 month or older rats for old subjects (Anversa, Li, Sonnenblick, & Olivetti, 1994; Boluyt, Converso, Hwang, Mikkor, & Russell, 2004; Delp, Evans, & Duan, 1998; Pacher, et al., 2004). In comparing to humans, the 3-6 month old rat would be comparable in structure and function to a 15-20 year old human, while a 24+ month old rat would be comparable to a 60+ year old human (Pacher, et al., 2004).

Another interesting model of animal aging is the caloric restriction model. It has been found that moderate caloric restriction in aged rats improved markers of normal aging such as serum prostaglandins (Choi & Yu, 1998), cerebral blood flow (Lynch, et al., 1999), renal damage and failure (Jiang, Liebman, Lucia, Phillips, & Levi, 2005), and expression of VCAMs (Zou, et al., 2006). Many aging model rats such as the F344 strain are subjected to a calorie restricted diet, which significantly extends lifespan in these animals. The precise mechanisms responsible for enhancing lifespan through caloric restriction are not fully understood, but could involve an AMPK or SIRT1 pathway since treatment with resveratrol has been shown to mimic the effects of caloric restriction (Pearson, et al., 2008).

2. Rationale, Objectives & Hypotheses

2.1: Summary of Rationale

Previous data has clearly demonstrated that the vascular adventitia is a dynamic tissue capable of mediating many processes, both in health and disease (An, et al., 2006; Dourron, et al., 2005; Garanich, et al., 2007; Kleschyov, et al., 1998; Laflamme, et al., 2006; Siow & Churchman, 2007; Zhang, Du, Cohen, Chobanian, & Brecher, 1999). The vascular adventitia may directly affect and alter the structure and function of the vasculature on a whole, and can no longer be considered a passive structural support of the vessel wall. The process of vascular aging is commonly associated with structural and functional changes in the vessel wall, including hypertrophy (Briones, et al., 2007), cell proliferation and apoptosis (Torella, et al., 2004), and ECM deposition (Greenwald, 2007). Similarly, aging is also associated with a tremendous increase in oxidative stress (Csiszar, et al., 2002), which serves to mediate numerous age-related vascular disorders. However, the specific role that the adventitia may play in the aging vasculature is still unknown. The purpose of this study will be to determine the possible role(s) that the adventitia may play in the age related changes of the structure and function of the vasculature.

2.2: Objectives & Hypotheses

Main Hypothesis: The vascular adventitia plays a significant role in the mediation of age-related changes in the vasculature. Chief among these changes is the up-regulation of key ET system proteins, which presumably would result in enhanced expression of the

potent vasoactive and mitogenic peptide ET-1. The enhanced expression of ET-1 would then result in vascular wall hypertrophy, increased ECM deposition, and enhanced cell proliferation. Additionally, since we have previously demonstrated the production of ET-1 to be dependent on a ROS signalling pathway (An, et al., 2007), we would expect an increase in overall oxidative stress in the vascular wall with advancing age. I hypothesize that the vascular adventitia plays a large role in the mediation of each of these processes through its propensity for producing ET-1.

2.2.1: Objective 1

Determine the effect of age on vascular adventitia morphology and thickness.

1. Determine the changes in cell morphology in the vascular wall and adventitia of the rat aorta in different age groups.

   Rationale: Adventitial fibroblasts commonly undergo phenotypic switches to myofibroblasts in injury and disease states (Shi-Wen, et al., 2001; Shi, O'Brien, et al., 1996; Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002). These changes can often be measured through characteristic morphological changes. During aging, blood vessels commonly are subjected to numerous injurious stimuli, thus requiring the presence of myofibroblasts for remodelling.

   Methods: These changes will be viewed through a haematoxylin and eosin staining procedure on each of the 15, 30, 50 and 80 week old rat aorta sections.

   Prediction: It is my hypothesis that the presence of morphologically distinct myofibroblasts will be greatly enhanced among aged animals.

2. Determine the changes in vascular wall and adventitial thickness of the rat aorta

with aging.

Rationale: As has been known for years, blood pressure steadily increases with age in most individuals. Along with this increase in blood pressure comes a stiffening of the arteries, caused by increased ECM deposition (Izzo & Mitchell, 2007). Similarly, there may be an associated vascular cell hypertrophy, resulting in thicker blood vessels (Briones, et al., 2007).

Methods: This aspect can be ascertained through haematoxylin and eosin staining of the 15, 30, 50 and 80 week old rat aorta sections and measuring the area and thickness of the vascular wall and adventitia.

Prediction: It is my hypothesis that the area and thickness of the adventitia and vascular wall will be greater among older animals.

2.2.2: Objective 2

Determine the effect of age on vascular adventitial cell hypertrophy, proliferation, and apoptosis with aging.

1. Determine the degree of cell hypertrophy in the vascular wall and adventitia of the rat aorta in different age groups.

   Rationale: Numerous sources have described an enhanced intima-media thickness in the blood vessels of aged subjects (Briones, et al., 2007; Moreau, et al., 1998; Tanaka, et al., 2001). Although these studies have not addressed the adventitia specifically, one would expect similar growth responses to occur in the adventitia. Thus, it would be expected that this enhanced thickening of the vessel wall may be partially due to cell hypertrophy both in the adventitia and the rest of

the vessel wall.

Methods: Similar to the determination of vascular wall thickness and area augmentation with aging, the vascular cell hypertrophy can be determined by the ratio of cell mass area to the number of nuclei stained by haematoxylin and eosin staining. This will yield a measure of the average size of vascular cells in each of the 15, 30, 50 and 80 week old rat aorta sections.

Prediction: It is my hypothesis that the average size of vascular and adventitial cells will increase dramatically with age.

2. Determine the degree of vascular cell proliferation in the vascular wall and adventitia of the rat aorta in different age groups.

   Rationale: Vascular cell proliferation is a common hallmark of many cardiovascular diseases (Barandier, et al., 2005; Das, et al., 2001). Since aging is associated with increases in numerous cardiovascular diseases, it would be reasonable to surmise that vascular cell proliferation (including the adventitia) would be enhanced in aging.

   Methods: In order to test this, the expression of Ki67 (a protein expressed only in actively proliferating cells) in the vascular adventitia of rat aorta sections isolated from 15, 30, 50 and 80 week old animals will be examined by immunofluorescence.

   Prediction: It is my hypothesis that Ki67 expression will closely correlate with aging.

3. Determine the degree of cellular apoptosis in the vascular wall and adventitia of the rat aorta in different age groups.

Rationale: Along with vascular cell proliferation, vascular cell turnover is important in the progression of cardiovascular diseases (Desmouliere, Badid, Bochaton-Piallat, & Gabbiani, 1997; Intengan & Schiffrin, 2001). One marker for cell turnover rate is the amount of apoptotic cells present.

Methods: Thus, the expression of cleaved Caspase-3 protein, a specific marker of apoptosis, will be examined in the vascular adventitia of rat aorta sections isolated from 15, 30, 50 and 80 week old animals by immunofluorescence.

Prediction: It is my hypothesis that the degree of apoptosis will closely correlate with aging.

2.2.3: Objective 3

Determine the effect of age on the expression of the ET system in the vascular adventitia *in vivo*.

1. Determine the expression of the ET system in the vascular adventitia of rat aorta in different age groups.

   Rationale: It has been shown that the ET-1 peptide can cause contraction of a tissue engineered adventitial blood vessel (Laflamme, et al., 2006). Additionally, we have previously shown that ET-1 is expressed and released by cultured mouse aortic adventitial fibroblasts (An, et al., 2006), however a functional ET system has yet to be demonstrated in the adventitia *in vivo*. The aorta serves as an ideal location for examining changes in the adventitia since the aorta yields the largest amount of adventitial cell mass of any vessel (Greenwald, 2007). Thus, changes in the adventitia during aging will be most easily detected in the aorta.

Methods: In order to accomplish this objective, aorta sections isolated from 15, 30, 50 and 80 week old animals will be examined by immunofluorescence.

Prediction: It is my hypothesis that the ET system proteins are all present in the aortic adventitia.

2. Determine the expression of the ET system in the vascular adventitia of rat mesenteric arteries in different age groups.

   Rationale: Although the aorta serves as a great source of adventitial cell mass, the smaller muscular arteries are considered to be more important in mediating systemic hypertension (Schiffrin, 2003). Additionally, there is some concern that the cellular phenotypes observed in the aorta are not consistent with those observed in other vascular beds.

   Methods: Thus, the expression of the ET system in mesenteric artery sections isolated from 15, 30, 50 and 80 week old animals will be examined by immunofluorescence.

   Prediction: It is my hypothesis that the ET system proteins are all expressed in the mesenteric artery adventitia.

3. Determine the expression of the ET system in the vascular adventitia of rat pudendal arteries in different age groups.

   Rationale: Further to the above objective, the pudendal artery represents a smaller vessel than the mesenteric arteries, and thus may better approximate the biology of small resistance vessels (Intengan & Schiffrin, 2000). By examining these vessels, we can gain a better understanding of regional differences in vascular adventitia biology.

Methods: The expression of the ET system in pudendal artery sections isolated from 15, 30, 50 and 80 week old animals will be examined by immunofluorescence.

Prediction: It is my hypothesis that the ET system proteins are all expressed in the pudendal artery adventitia.

2.2.4: Objective 4

Determine the downstream intracellular effects of ET-1 in the adventitia in different age groups in regards to ROS production, ECM deposition, and myofibroblast induction.

1. Determine the accumulated effects of ROS production in the vascular adventitia of the rat aorta in different age groups.

   Rationale: We have previously shown that the production and release of ET-1 in cultured mouse aortic adventitial fibroblasts follows an NADPH oxidase/$^{\cdot}O_2^-$-dependent pathway (An, et al., 2007). Additionally, ROS production in aging is tremendously important, mediating vascular hypertrophy (Liu, et al., 2004), cell migration (Haurani, et al., 2008), and injury repair (Shi, O'Brien, et al., 1996). With the capability to produce copious ROS, the adventitia is particularly important in aging for its ability to mediate each of these effects.

   Methods: As such, we may be able to detect the intracellular marker of this increased $^{\cdot}O_2^-$ production, namely 3-nitrotyrosine. Thus, the expression of 3-nitrotyrosine in the vascular adventitia of rat aorta sections isolated from 15, 30, 50 and 80 week old animals will be examined by immunofluorescence.

   Prediction: It is my hypothesis that the expression of 3-nitrotyrosine will

correlate closely to the expression of the ET system in these sections.

2. Determine the ECM deposition in the vascular adventitia of the rat aorta in different age groups.

   Rationale: The process of vascular aging is strongly influenced by ECM deposition, with matrix proteins serving as scaffolding for cell migration(Greiling & Clark, 1997; Rocnik, et al., 1998), as well as stiffening the vessel wall (Greenwald, 2007). We have additionally previously shown that ET-1 stimulation in cultured mouse aortic adventitial fibroblasts results in the synthesis of collagen type I protein, a major component of the vascular ECM (An, et al., 2007). Fibronectin is also a major component of the vascular ECM and has been shown to be an important mediator of fibroblast wound repair (Greiling & Clark, 1997; A. W. Hahn, et al., 1993).

   Methods: Thus, the expression of fibronectin and collagen type I in the vascular adventitia of rat aorta sections isolated from 15, 30, 50 and 80 week old animals will be examined by immnuofluorescence.

   Prediction: It is my hypothesis that the expression of fibronectin and collagen will also closely correlate to the expression of the ET system in these sections.

3. Determine the degree of myofibroblast induction in the vascular adventitia of the rat aorta in different age groups.

   Rationale: Along with ET-1 expression, ROS production and ECM deposition, it has been hypothesized that myofibroblasts are important in mediating cardiovascular diseases common among aged populations (Xu, Ji, Li, Chen, & Hu, 2007). Myofibroblasts are often involved in injury repair processes, and their

presence may result in enhanced vascular stiffness (Gabbiani, 2003).

Methods: To determine their presence in the vasculature, the expression of α-SMA (a marker of myofibroblasts) in the vascular adventitia of rat aorta sections isolated from 15, 30, 50 and 80 week old animals will be examined by immunofluorescence.

Prediction: It is my hypothesis that the degree of myofibroblast induction will correlate to the expression of the ET system in these sections.

3: Methodology

3.1: Animals and Tissue Preparation

The animal and tissue preparation procedures described below were performed by the lab of Dr. Michael A. Adams at Queen's University, Kingston, Ontario, Canada. All animal protocols were approved and conducted according to the recommendations from the Research Sub-Committee of Queen's University on Animal Care and Use and the Canadian Council on Animal Care. Male Sprague-Dawley rats were obtained from Charles River Laboratories (Toronto, ON). Sprague-Dawley rats were chosen over an aging strain such as the F344 strain to provide a more natural aging process, which would be unaltered by selective breeding or caloric restriction. Rats were housed individually at a temperature of 22-24°C with a 12hr light/dark cycle. Rats were provided with regular rodent chow (LabDiet® 5001, Ren's Feed and Supply Ltd., Oakville, ON) and tap water *ad libitum*. Blood pressure measurements were recorded through the use of implanted radio-telemetry units. At 15, 30, 50, and 80 weeks, animals were anesthetised with sodium pentobarbital (65mg/kg, intraperitoneally) followed by intravenous doses of the ganglionic blocker hexamethonium and heparin (30mg/kg and 1000 units/kg, respectively) to allow for perfusion under maximally relaxed conditions. After the heart was excised, the animals were perfused at 70mmHg through the thoracic aorta with saline (0.9% NaCl) containing the vasodilator sodium nitroprusside (300mg/L) to facilitate flushing out the blood. The animals were then perfusion fixed with 4% paraformaldehyde in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 2 mM KH_2PO_4, pH 7.4; PBS). Thoracic aortas, mesenteric arteries, and pudendal arteries were

excised, cleaned of extraneous connective tissue and perivascular fat, and stored in fixative overnight. These vessels were chosen for analysis for their relative ease of isolation as well as providing a representation of elastic conduit arteries (aorta), muscular arteries (mesenteric), and small resistance vessels (pudendal). In addition, these vessels also provide an estimation of regional differences in the vasculature since each is isolated from a separate and distinct region of the body. Vessels were then processed routinely for paraffin embedding (1 hr each in graded ethanols [70% x2, 95% x2, 100% x3], xylene x2, paraffin x3). Vessels were cut into 5μm sections and placed on glass slides.

3.2: Hematoxylin & Eosin Staining

Paraffin embedded tissues were de-waxed in 2-5 minute washes in xylene and rehydrated in graded ethanols (2x1 min in 100%, 2x1 min in 95%). Samples were fixed in formalin (Sigma) for 5 minutes at room temperature and then rinsed in PBS. Slides were stained in hematoxylin (Sigma) for 5 minutes, rinsed in tap water, stained in eosin (Sigma) for 5 minutes, and rinsed again in tap water. Samples were dehydrated in an ethanol gradient, and mounted with Permount Mounting Medium (Fisher) and a coverslip. The mounting medium was allowed to cure for approximately 24 hours before analysis, after which point slides were stored at room temperature. Slides were viewed under a Nikon light microscope and photomicrographs were taken under the 40X objective lens. Images were analyzed for structural and morphological measurements using ImageJ software (NIH).

3.3: Immunofluorescence Staining

Paraffin embedded tissues were de-waxed and rehydrated as described above. Sections were then fixed for 15 minutes in acetone/methanol (50/50 vol/vol) at 4°C. After rinsing in PBS twice for 5 minutes each, sections were permeabilized in 0.1% Triton X-100 (Sigma) in PBS for 5 min at room temperature. Sections were further rinsed in PBS twice for 5 minutes each, and blocked in 10% donkey serum (Sigma) in PBS for 1 hour at room temperature. Samples were incubated in 1° antibodies at 1:100 dilution overnight at 4C in 1% donkey serum in PBS. Samples were rinsed in PBS 3 times for 5 minutes each and incubated with the 2° antibodies at 1:400 dilution in PBS for 1 hour at room temperature, while being maintained in the dark until analysis. Samples were then rinsed in PBS 3 times for 5 minutes each and mounted with a cover slip using Prolong Gold antifade reagent with DAPI (Invitrogen). The mounting medium was allowed to cure at room temperature for approximately 24 hours before analysis, after which point samples were stored at 4°C. Slides were viewed under a Nikon fluorescent microscope, and photomicrographs were taken with a 40X objective lens at 4 representative areas per section. Images were analyzed for the area and intensity of staining, as well as for structural and morphological measurements using ImageJ software (NIH).

3.4 Image Analysis and Quantification

Images used for structural and morphological measurements were examined using ImageJ software (NIH). The thickness of the vessel wall was determined by measuring the distance from the external edge of the lumen to the external edge of the adventitia in a direction perpendicular to the vessel wall. This measurement was performed in

quadruplicate for each quadrant of the vessel wall and then averaged. The thickness of the adventitia was determined by measuring the distance from the external elastic lamina to the external edge of the adventitia along the same line used to measure the vessel wall. Similarly this measurement was performed in quadruplicate for each quadrant of the vessel wall and then averaged. The thickness of the medial layer was determined by subtracting each adventitia thickness measurement from the corresponding vessel wall thickness measurement which were determined as described above. As with the previous measurements, the thickness of the medial layer was measured in quadruplicate for each quadrant of the vessel wall and then averaged. Since vessels were not uniformly patent for all samples, direct measurement of lumen diameter was not possible. Thus, the lumen border of each vessel was traced and measured to determine the lumen circumference which was then divided by pi to yield lumen diameter. This measurement was performed in quadruplicate for each vessel, and then averaged.

Images used for quantifying immunofluorescence staining were analyzed by ImageJ software (NIH). Immunofluorescence photomicrographs consisted of a stacked three channel RGB image, comprising the DAPI (blue), FITC (green), and TRITC (red) channels. To determine immunofluorescent staining in the adventitial layer, the entire area comprising the adventitia was highlighted using a tracing tool. The image was then broken into individual grayscale images, with one image corresponding to each of the DAPI, FITC, and TRITC channels. The individual grayscale images were then thresholded to identify positive staining. Each image was then measured for the total area of positive staining in the highlighted region, and in the case of DAPI staining, the number of individual objects (nuclei) were counted. The same procedure was repeated to

determine staining in the medial layer after highlighting the medial layer. Total area measurements were also taken for the adventitia and media, regardless of positive staining. The positive staining measurements were then divided by the total area measurements for their corresponding wall layer to yield a measure of positive immunofluorescent staining expressed as a percentage of the total layer area. The total area measurements were also used to calculate cell size in each sample by dividing the total area measurement by the total number of nuclei present in the corresponding wall layer. The method for analyzing immunofluorescent images is shown below in Figure 4.

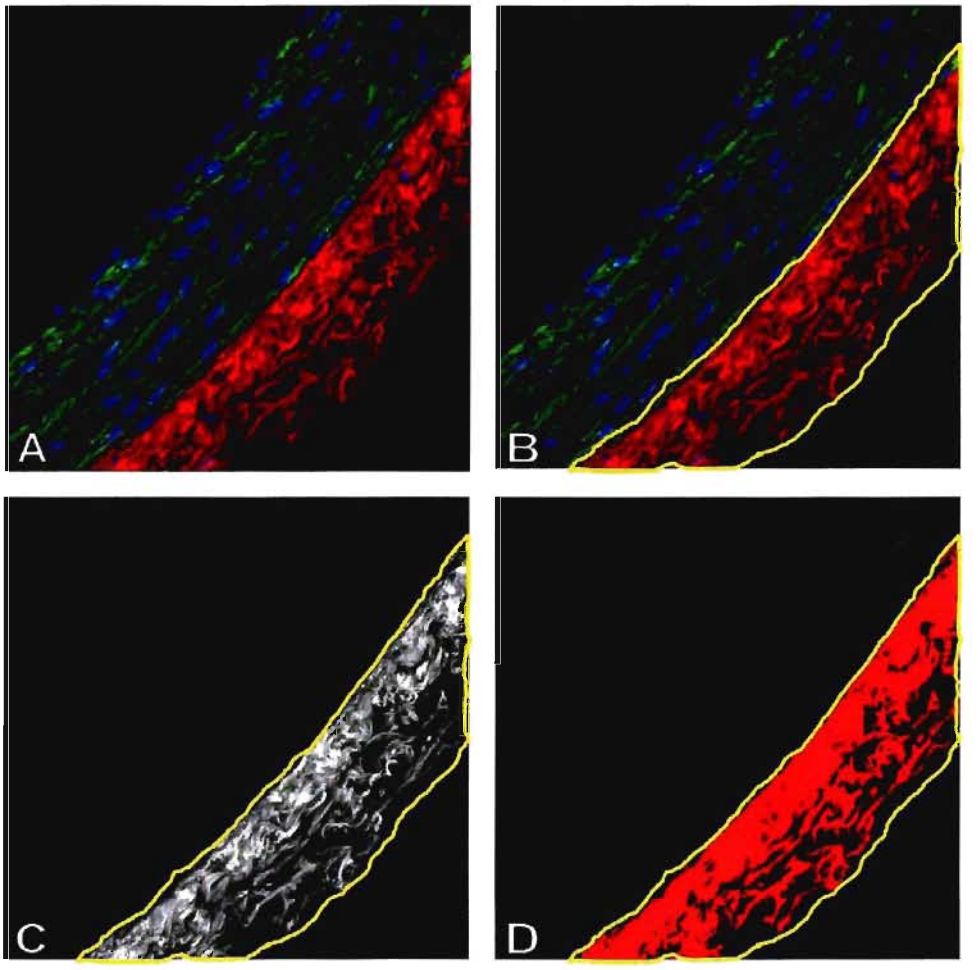

Figure 4. Method for analyzing immunofluorescent images. Immunofluorescence photomicrographs consisted of a stacked three channel RGB image, comprising the DAPI (blue), FITC (green), and TRITC (red) channels (A). To determine immunofluorescent staining in the adventitial layer, the entire area comprising the adventitia was highlighted using a tracing tool (B). The image was then broken into individual grayscale images, with one image corresponding to each of the DAPI, FITC, and TRITC channels (C – only TRITC channel shown). The individual grayscale images were then thresholded to identify positive staining, denoted in red (D). Each image was then measured for the total area of positive staining in the highlighted region. The process is repeated for the medial layer.

3.5: Antibodies and Chemicals Used

Primary antibodies used were goat collagen type I polyclonal antibody (Santa

Cruz sc8784), rabbit ET_A polyclonal antibody (Chemicon International AB32860), rabbit ET_B polyclonal antibody (Chemicon International AB3284), rabbit α-SMA polyclonal antibody (Abcam ab5694) rabbit fibronectin polyclonal antibody (Abcam ab299), goat ECE-1 polyclonal antibody (Santa Cruz sc27557), goat laminin polyclonal antibody (Santa Cruz sc16582), rabbit ET-1 polyclonal antibody (Abbiotec 250633), rabbit Ki67 polyclonal antibody (Abcam ab66155), rabbit 3-nitrotyrosine polyclonal antibody (Invitrogen A21285), rabbit von Willebrand Factor polyclonal antibody (Abcam ab6994), rabbit cleaved Caspase-3 polyclonal antibody (Cell Signalling 9661S). Secondary antibodies used were donkey anti-rabbit Alexa Fluor 488 (Invitrogen A21206) and donkey anti-goat Alexa Fluor 546 (Invitrogen A11056). Mounting medium was ProLong Gold antifade with DAPI (Invitrogen P36931) for immunofluorescence and Permount Mounting Medium (Fisher SP15-100) for haematoxylin and eosin staining. Other reagents used for tissue analysis were Donkey serum (Sigma D9663), Triton X-100 (Sigma T9284) p-Xylene (Sigma 95682), Acetone (Sigma 534064), Methanol (Sigma M3641) Formalin (Sigma 11-0705), Haematoxylin (Sigma H3136), Eosin Y (Sigma E4009).

3.6: Statistics

Data are expressed as mean ± SEM. Graphic and statistical analyses were performed using GraphPad Prism 5.0 software. Analysis of protein expression levels, vessel measurements, and cell size in samples with more than two groups were performed using one-way ANOVA with Tukey's post hoc test. Analysis of protein expression levels, vessel measurements, and cell size in samples with two groups were performed

using Student's t-test. Differences were accepted as statistically significant at a p-value <0.05.

4: Results

4.1 Vascular Adventitia Morphology and Vessel Wall Thickness

4.1.1 Vascular Adventitia Morphology

As evidenced from histological examination of the tissues, the vascular wall changes dramatically with aging. The 15 week old aorta sections display a clean and organized vessel wall, with clearly demarcated elastic laminas between layers of medial VSMCs. The adventitial region of the 15 week old aortas is also relatively small and compact compared to its older counterparts. Overt morphological changes are apparent as early as 30 weeks of age, with aorta sections displaying disorganized elastic laminas, larger VSMCs, and an expansion of the adventitia. As the aorta increases in age, the degree of cellular disorganization increases as well, and the 80 week old aorta sections display a vastly different morphology to their 15 week old counterparts. A similar relationship is found in the mesenteric arteries, with the 15 week old controls displaying a relatively small and organized vessel wall structure compared to their older counterparts. At 80 weeks of age, the mesenteric artery wall appears vastly different from that of the 15 week old controls. This relationship is most apparent in the pudendal artery sections, with the 15 week old controls displaying a similarly clean and organized vessel wall compared to its 80 week old counterparts.

In addition, the vascular adventitia of each vessel type is most likely comprised primarily of fibroblasts, since cell marker analysis displayed minimal expression of von Willebrand Factor (ECs; Figure A-2), CD4 (macrophages; Figure A-3), c-kit (stem cells;

Figure A-4), and α-SMA (VSMCs; Figures 43 & 45) in the adventitia.

4.1.2 Vessel Wall Morphometry

4.1.2.1 Aortic Wall Morphometry

The key morphometric measurements of the aortic wall are presented below in Table 1. As expected, the thickness of the aortic wall increased dramatically with increasing age, with the 80 week old aortas displaying approximately a 75% increase over the 15 week old aortas (Table 1). Both the medial and adventitial layers increased in thickness, first reaching significance at 30 and 50 weeks for the media and adventitia, respectively. The increase in aortic wall thickness progressed consistently thereafter until 80 weeks of age. At each time point, the medial layer was significantly thicker than its corresponding adventitia (Table 1). Aortic cross sectional area (CSA) showed a similar relationship, with total wall CSA increasing progressively with increasing age, and medial CSA always remaining more substantial than its corresponding adventitia CSA (Table 1).

Table 1. Morphometric measurements of the aortic wall.

Measurement	*15 wks*	*30 wks*	*50 wks*	*80 wks*
Wall Thickness (μm)	$224.6 \pm 6.3^{c,d}$	$253.6 \pm 5.6^{a,d}$	$258.7 \pm 5.7^{a,d}$	$342.6 \pm 4.4^{a,b,c}$
Media Thickness (μm)	$133.0 \pm 2.6^{b,c,d,e}$	$157.4 \pm 2.7^{a,d,e}$	$151.8 \pm 1.9^{a,d,e}$	$223.1 \pm 2.5^{a,b,c,e}$
Adventitia Thickness (μm)	$91.5 \pm 4.7^{c,d}$	96.2 ± 3.8^{d}	106.8 ± 4.6^{a}	$119.4 \pm 2.9^{a,b}$
Adventitia:Media Ratio (%)	65.6 ± 3.6	57.2 ± 3.4	66.8 ± 3.6	54.1 ± 1.9
Wall CSA (x 10^3 μm^2)	$1044.1 \pm 64.5^{b,c,d}$	$1397.4 \pm 88.7^{a,d}$	$1532.3 \pm 71.4^{a,d}$	$2198.7 \pm 31.4^{a,b,c}$
Media CSA (x 10^3 μm^2)	$593.0 \pm 28.6^{b,c,d,e}$	$834.7 \pm 35.9^{a,d,e}$	$866.4 \pm 30.2^{a,d,e}$	$1341.7 \pm 23.0^{a,b,c,e}$
Adventitia CSA (x 10^3 μm^2)	$451.0 \pm 39.9^{c,d}$	562.7 ± 55.0^{d}	$665.8 \pm 48.9^{a,d}$	$857.0 \pm 25.0^{a,b,c}$
Lumen Diameter (μm)	$1347.1 \pm 14.3^{b,c,d}$	$1526.7 \pm 13.6^{a,c,d}$	$1611.7 \pm 21.6^{a,b}$	$1657.0 \pm 18.7^{a,b}$
Wall:Lumen Ratio (%)	15.7 ± 2.2^{d}	16.2 ± 2.5^{d}	16.1 ± 1.8^{d}	$21.1 \pm 2.0^{a,b,c}$

Mean ± SEM; [a]$p<0.05$ vs. 15 wks; [b]$p<0.05$ vs. 30 wks; [c]$p<0.05$ vs. 50 wks; [d]$p<0.05$ vs. 80 wks, [e]$p<0.05$ vs. corresponding adventitia

Despite the media consistently being thicker than its corresponding adventitia, the increase in adventitial thickness is paralleled by the increase in medial thickness with age as evidenced by the adventitia to media thickness ratio (Table 1). From 15 weeks to 80 weeks there was no observed change in adventitia to media thickness ratio, suggesting the adventitia is growing at a similar rate to the media with increasing age.

From 15 to 50 weeks of age, the growth in the aortic wall thickness occurred at a pace that matched the advancing growth in lumen diameter (Table 1). However, at 80 weeks of age, aortic wall thickness grew beyond the pace set by lumen diameter (Table 1).

4.1.2.2 Mesenteric Artery Wall Morphometry

The key morphometric measurements of the mesenteric artery wall are presented below in Table 2. The mesenteric artery wall also expanded in thickness with increasing

age, however less consistently than in the aorta. Again, both the medial and adventitial layers of the vessel wall thickened with age, reaching significance at 30 and 80 weeks, respectively, when compared to the 15 week old controls (Table 2). Unlike the aortic wall, at all time points the mesenteric artery adventitial layer remained significantly thicker than its corresponding medial layer (Table 2). Similarly, total wall CSA increased from 15 to 30 weeks, before decreasing non-significantly at 50 weeks then increasing again at 80 weeks. At all time points, adventitial CSA was greater than its corresponding medial CSA.

Table 2. Morphometric measurements of the mesenteric artery wall.

Measurement	*15 wks*	*30 wks*	*50 wks*	*80 wks*
Wall Thickness (μm)	$85.9 \pm 6.0^{b,d}$	$115.3 \pm 4.1^{a,c,d}$	$76.3 \pm 3.1^{b,d}$	$139.0 \pm 5.4^{a,b,c}$
Media Thickness (μm)	$30.4 \pm 1.7^{b,d,e}$	$35.9 \pm 0.9^{a,c,d,e}$	$29.2 \pm 0.5^{b,d,e}$	$67.3 \pm 2.2^{a,b,c,e}$
Adventitia Thickness (μm)	$55.4 \pm 4.1^{b,d}$	$79.3 \pm 3.5^{a,d}$	$45.9 \pm 2.6^{b,d}$	$79.6 \pm 2.9^{a,c}$
Adventitia:Media Ratio (%)	$184.8 \pm 11.0^{b,d}$	$224.7 \pm 10.1^{a,c,d}$	$155.5 \pm 7.9^{b,d}$	$120.3 \pm 3.5^{a,b,c}$
Wall CSA (x 10^3 μm^2)	$101.8 \pm 7.2^{b,c,d}$	$221.4 \pm 16.9^{a,d}$	$193.4 \pm 12.9^{a,d}$	$327.2 \pm 24.5^{a,b,c}$
Media CSA (x 10^3 μm^2)	$41.2 \pm 4.1^{b,c,d,e}$	$65.7 \pm 3.9^{a,d,e}$	$63.8 \pm 3.9^{a,d,e}$	$137.2 \pm 8.2^{a,b,c,e}$
Adventitia CSA (x 10^3 μm^2)	$60.6 \pm 4.6^{b,c,d}$	155.6 ± 15.7^{a}	$129.5 \pm 11.3^{a,d}$	$190.0 \pm 17.8^{a,c}$
Lumen Diameter (μm)	$504.6 \pm 21.3^{c,d}$	$522.3 \pm 16.3^{c,d}$	$635.6 \pm 19.7^{a,b}$	$653.7 \pm 19.0^{a,b}$
Wall:Lumen Ratio (%)	$11.7 \pm 3.3^{b,d}$	$21.3 \pm 4.1^{a,c}$	$13.5 \pm 2.9^{b,d}$	$20.8 \pm 6.2^{a,c}$

Mean ± SEM; $^{a}p<0.05$ vs. 15 wks; $^{b}p<0.05$ vs. 30 wks; $^{c}p<0.05$ vs. 50 wks; $^{d}p<0.05$ vs. 80 wks, $^{e}p<0.05$ vs. corresponding adventitia

In the case of the mesenteric arteries, the increase in adventitial wall thickness did not match the increase in medial wall thickness with increasing age. Although at 30 weeks the adventitia to media thickness ratio is actually greater than at 15 weeks, the relationship then turns downward with significant decreases between 30 and 50 weeks, as

well as between 50 and 80 weeks (Table 2).

The mesenteric arteries display a biphasic relationship between age and wall to lumen ratio. Both the 30 and 80 week old groups display a wall to lumen ratio that is significantly greater than that displayed by both the 15 and 50 week old groups (Table 2).

4.1.2.3 Pudendal Artery Wall Morphometry

The key morphometric measurements of the pudendal artery wall are presented below in Table 3. Like the aortic and mesenteric samples, the pudendal artery sections displayed an increase in overall wall thickness with increasing age. Although the adventitial layer displayed no significant change in thickness from 15 to 80 weeks, the medial layer thickness more than tripled between 15 and 80 weeks (Table 3). Interestingly, the media was significantly thinner than the adventitia at 15 weeks of age, but grew to be significantly thicker than the adventitia at 80 weeks of age (Table 3). Conversely, the adventitial CSA actually increased from 15 to 80 weeks, although not significantly.

Unsurprisingly, the pudendal artery adventitia to media thickness ratio also plummeted between 15 and 80 weeks of age, suggesting a much greater hypertrophy in the medial layer as compared to the adventitia (Table 3). Furthermore the pudendal arteries displayed a massive increase in wall to lumen ratio between 15 and 80 weeks of age (Table 3).

Table 3. Morphometric measurements of the pudendal artery wall.

Measurement	*15 wks*	*80 wks*
Wall Thickness (μm)	132.0 ± 10.6	239.9 ± 12.0[a]
Media Thickness (μm)	42.1 ± 2.7[b]	154.0 ± 6.7[a,b]
Adventitia Thickness (μm)	89.9 ± 9.1	85.9 ± 7.3
Adventitia:Media Ratio (%)	220.0 ± 18.5	56.3 ± 4.0[a]
Wall CSA (x 10^3 μm^2)	265.1 ± 50.9	521.3 ± 34.2[a]
Media CSA (x 10^3 μm^2)	71.1 ± 11.0[b]	310.6 ± 24.2[a,b]
Adventitia CSA (x 10^3 μm^2)	194.0 ± 41.5	210.7 ± 16.9
Lumen Diameter (μm)	476.1 ± 16.4	525.6 ± 12.2[a]
Wall:Lumen Ratio (%)	27.1 ± 3.1	42.1 ± 2.2[a]

Mean ± SEM; [a]$p<0.05$ vs. 15 wks, [b]$p<0.05$ vs. corresponding adventitia

4.2 Cell Hypertrophy, Proliferation and Apoptosis

4.2.1 Cell Hypertrophy

4.2.1.1 Aortic Cell Hypertrophy

The average size of the cells of the aortic adventitia increased substantially with age with each of the 30, 50, and 80 week old samples displaying significantly larger aortic adventitial cells than their 15 week old counterparts (Figure 5). Similarly, the cells of the aortic media also increased with age, following a similar pattern to the cells of the adventitia. However, the increase in medial cell size with age was far less dramatic than the increase in adventitial cell size. At each time point, the average size of adventitial cells was greater than that of their corresponding medial cells (Figure 5).

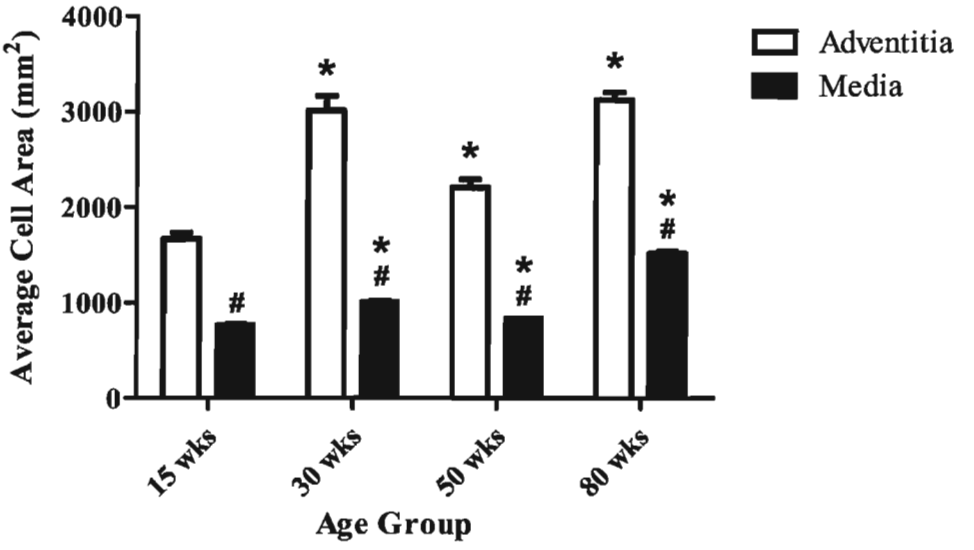

Figure 5. Average cell area in the aortic wall. Cells in the aortic adventitia displayed a great increase in size at 30, 50, and 80 weeks compared to 15 weeks (open bars), while medial cells showed a similar increase in overall size only at 30, 50 and 80 weeks compared to 15 weeks (filled bars). At each time point, the average size of adventitial cells was greater than that of the medial cells. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. *$p<0.05$ vs. 15wks group, #$p<0.05$ vs. corresponding adventitia; N=10 per group.

4.2.1.2 Mesenteric Artery Cell Hypertrophy

The average size of the cells in the mesenteric artery adventitia also increased substantially with age, with cells displaying a significant increase in average size at both 30 and 80 weeks as compared to the 15 week old controls (Figure 6). The mesenteric medial cells also grew in average size with increasing age, but far less dramatically than in the adventitia. The increase in medial cell size was significant only at 80 weeks of age, when compared to the 15 week old controls. As with the aortic cells, at all time points the average size of cells in the adventitia was greater than the average size of cells in the

media (Figure 6).

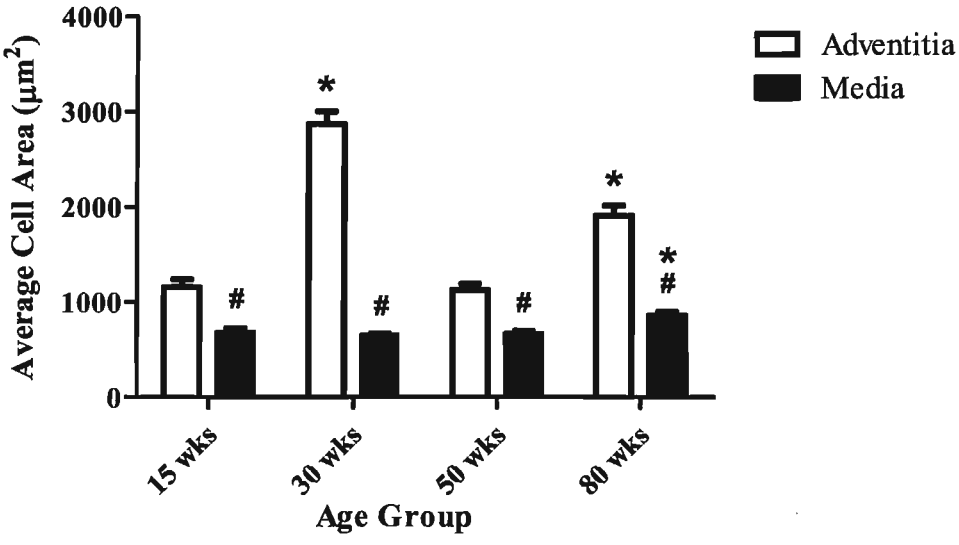

Figure 6. Average cell area in the mesenteric artery wall. Average cell size increased over time in both the adventitia (open bars) and media (filled bars). The average cell size was significantly greater in the adventitia at 30 and 80 weeks when compared to the 15 week old controls. Furthermore, the average cell size in the media was significantly greater at 80 weeks when compared to all other time points. At all time points, the average cell size was greater in the adventitial layer than in the medial layer. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. *$p<0.05$ vs. 15wks group, #$p<0.05$ vs. corresponding adventitia; N=8 per group.

4.2.1.3 Pudendal Artery Cell Hypertrophy

The average size of the cells in the pudendal artery increased slightly with age, significantly in the medial layer, but non-significantly in the adventitial layer (Figure 7). At each time point, the average size of cells in the adventitial layer was greater than the average size of cells in the medial layer (Figure 7).

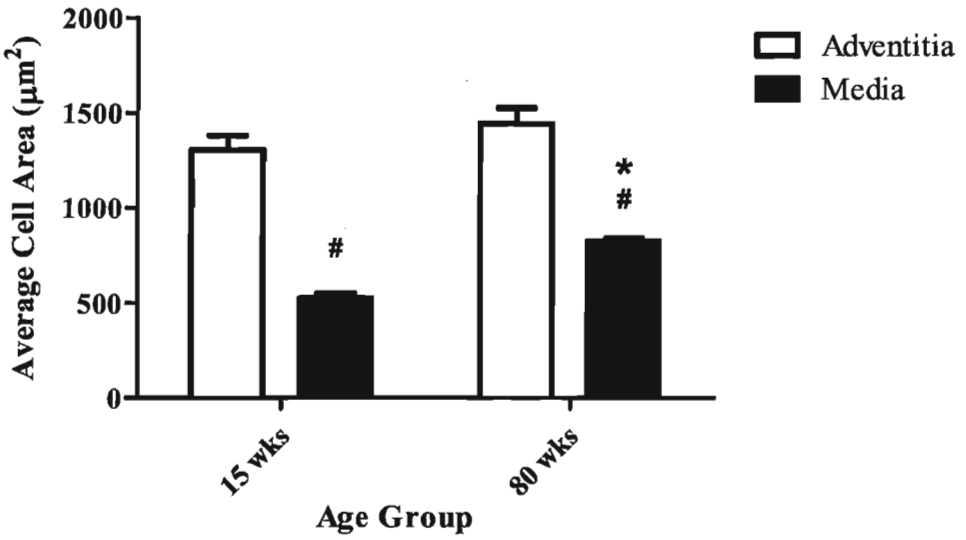

Figure 7. Average cell area in the pudendal artery wall. Average cell size increased significantly with increasing age in the media (filled bars), but remained unchanged in the adventitia (open bars). At each time point, the average cell size was greater in the adventitial layer than in the medial layer. Data are expressed as mean ± SEM. Data were analyzed by Student's t-test. *$p<0.05$ vs. 15wks group, #$p<0.05$ vs. corresponding adventitia; N=5-6 per group.

4.2.2 Ki67 Expression

Expression of the Ki67 cell proliferation marker in the aorta was almost entirely specific to the adventitial region at all ages (Figure 8).

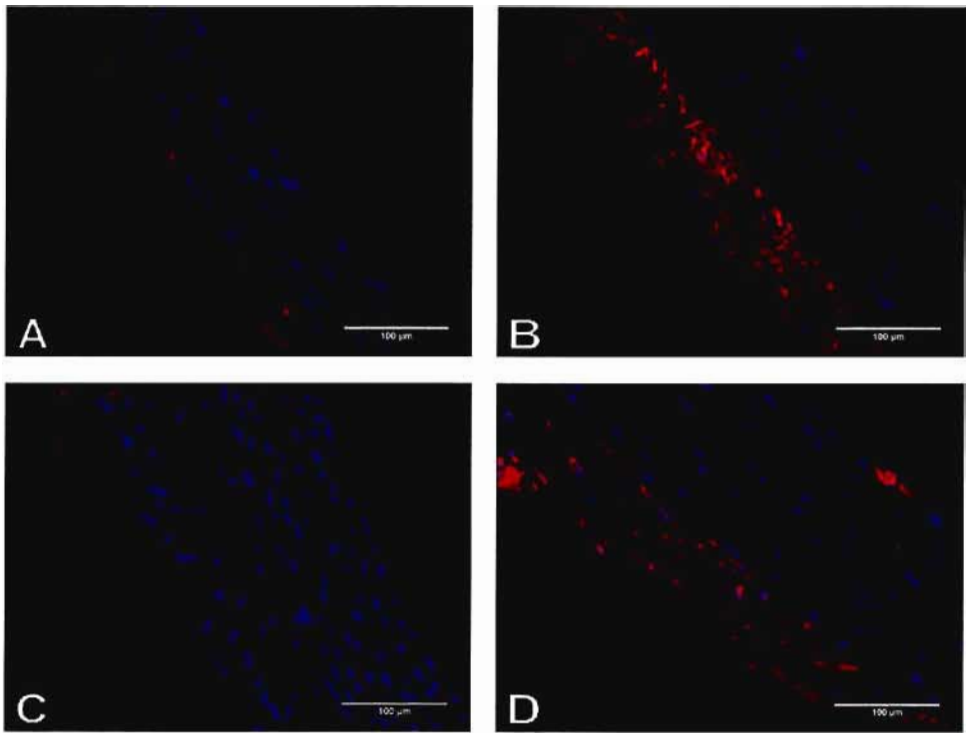

Figure 8. Ki67 protein localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C) and 80 weeks (D) all expressed Ki67 protein primarily in the adventitial layer. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5μm. Samples were then immunostained for Ki67 and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Zeiss fluorescent microscope with a 20X objective lens. Scale bars represent 100μm.

Quantification of Ki67 expression in the aorta revealed that the 30 and 80 week old aortas displayed significantly higher Ki67 expression in the adventitial layer compared to the 15 week old controls (Figure 9). In contrast, the Ki67 expression in the medial layer of the aorta showed a mild steady increase from ages 15 to 80 weeks, although not reaching significance. At 30 and 80 weeks of age, the relative expression of Ki67 was much greater in the adventitia compared to the media (Figure 9).

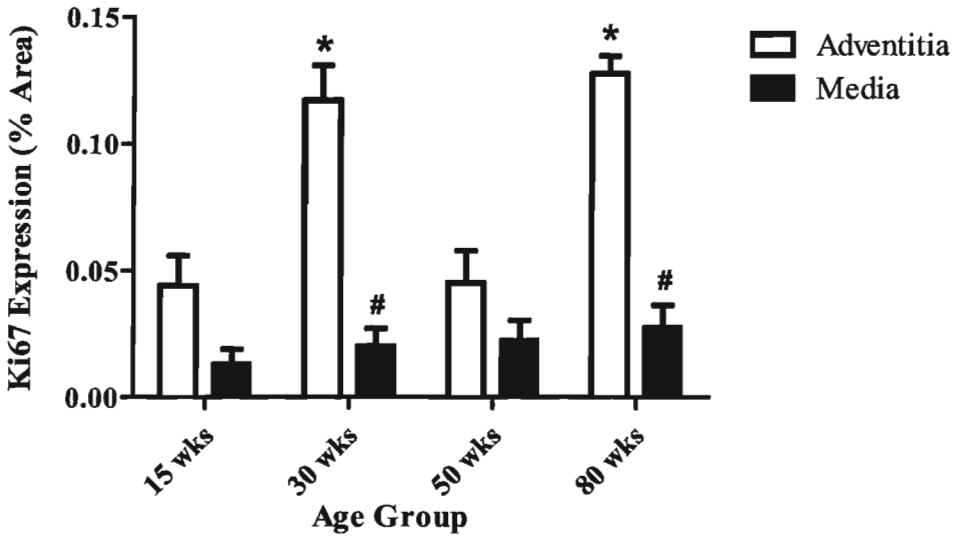

Figure 9. Quantitative analysis of Ki67 protein expression in the aortic wall. The aortic adventitia (open bars) displayed a significantly higher concentration of Ki67 than the medial layer (filled bars) at 30 and 80 weeks. Additionally, the adventitia displayed significantly greater Ki67 expression at 80 weeks when compared to 15 weeks. The aortic medial expression of Ki67 did not change with increasing age. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. *$p<0.05$ vs. 15wks group, #$p<0.05$ vs. corresponding adventitia; N=3-4 per group.

4.2.3 Caspase-3 Expression

Expression of the apoptotic signal cleaved caspase-3 displayed very little localization to the aortic wall in all age groups (Figure 10).

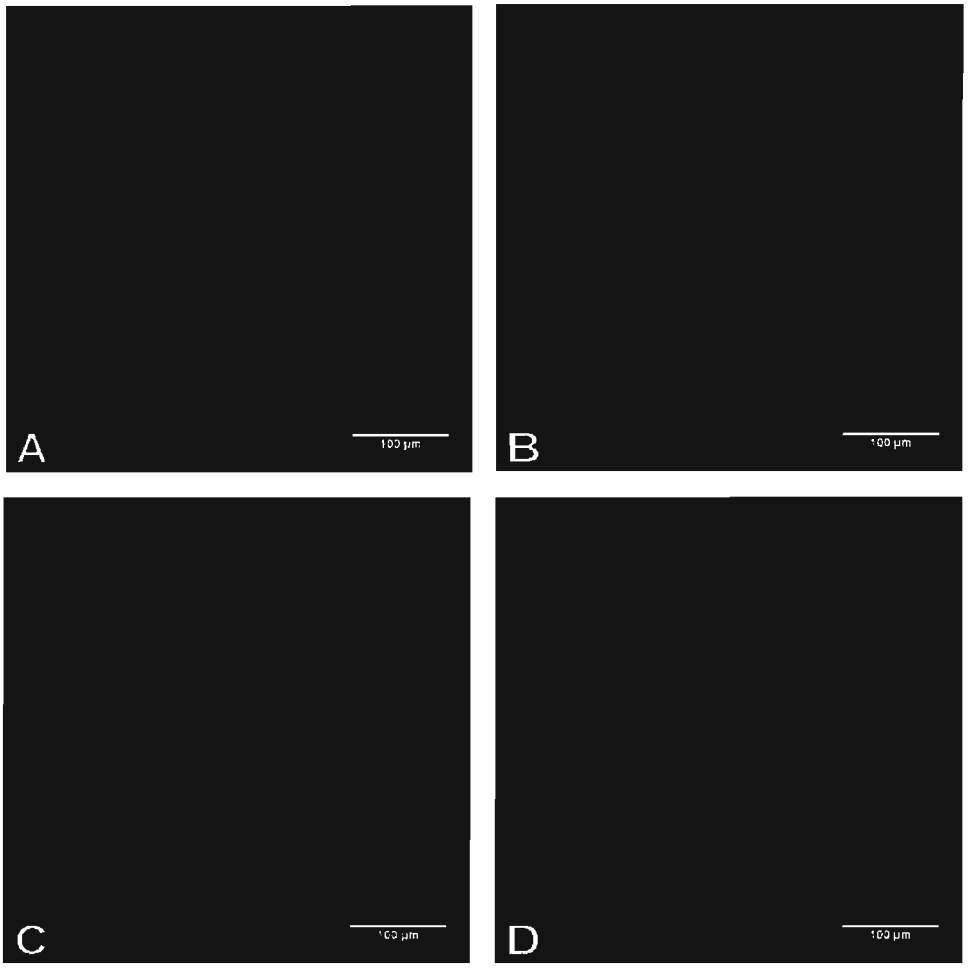

Figure 10. Cleaved caspase-3 protein localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C) and 80 weeks (D) expressed very little caspase-3 protein. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for cleaved caspase-3 and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Quantification of cleaved caspase-3 expression in the aorta revealed very low levels of expression throughout the aortic wall, but slightly more expression in the medial layer. The difference in expression between the adventitial and medial layers at all time

points was not significant (Figure 11).

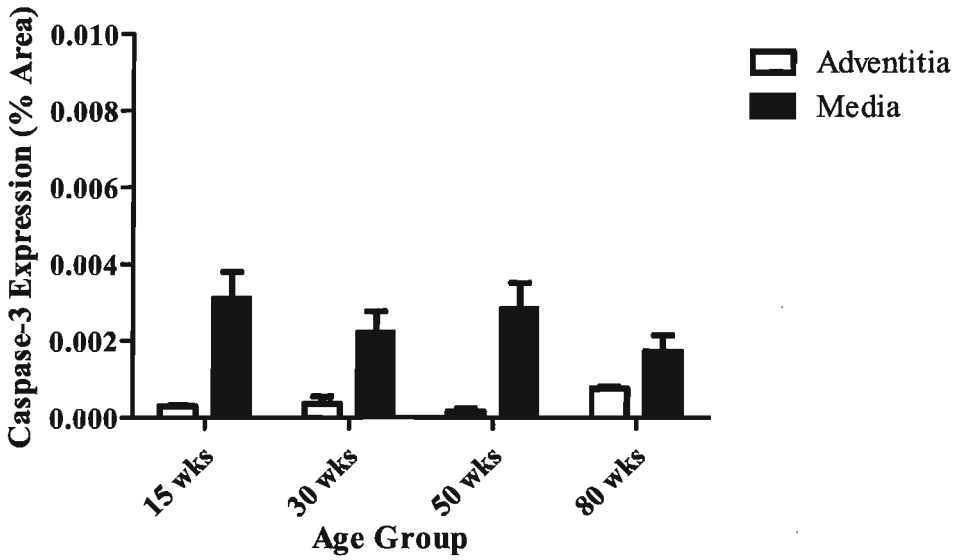

Figure 11. Quantitative analysis of caspase-3 protein expression in the aortic wall. The expression of caspase-3 did not change significantly with increasing age in either of the adventitial (open bars) or medial (filled bars) layers of the aortic wall. Expression was significantly greater in the medial layer of the 50 week old group compared to the adventitia, but did not differ in any other age group. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. N=4 per group.

4.3 Endothelin system expression

4.3.1 Aorta

4.3.1.1 ET-1

The expression of the ET-1 peptide in the aorta was not localized to any layer or compartment of the vessel wall, and as such ET-1 was found expressed throughout the vessel wall (Figure 12).

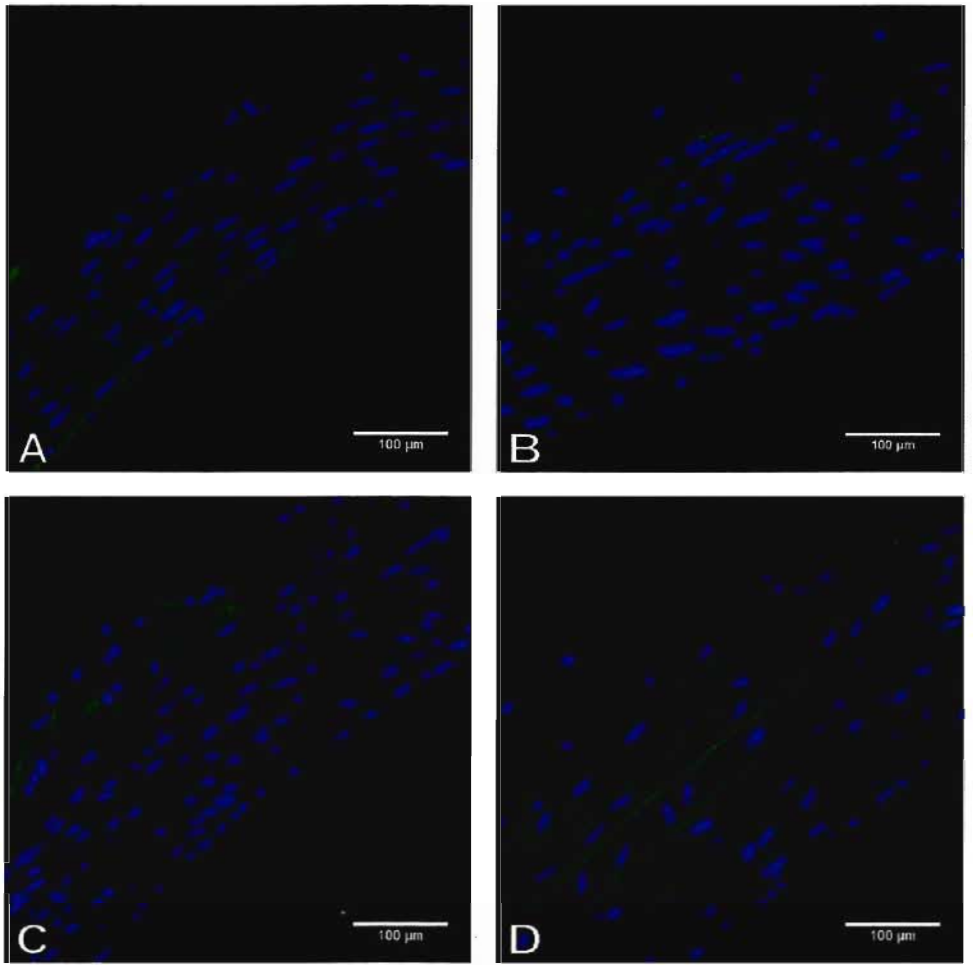

Figure 12. ET-1 protein localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C) and 80 weeks (D) all expressed ET-1 peptide throughout the vessel wall. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for ET-1 and detected with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Furthermore, quantitative analysis of ET-1 expression in the aorta revealed that expression in the adventitial layer was fairly low at all ages (Figure 13). The medial expression of the ET-1 peptide was consistent across all age groups, declining only

slightly with age, without reaching significance. At all time points the medial layer exhibited significantly greater expression than the adventitia (Figure 13).

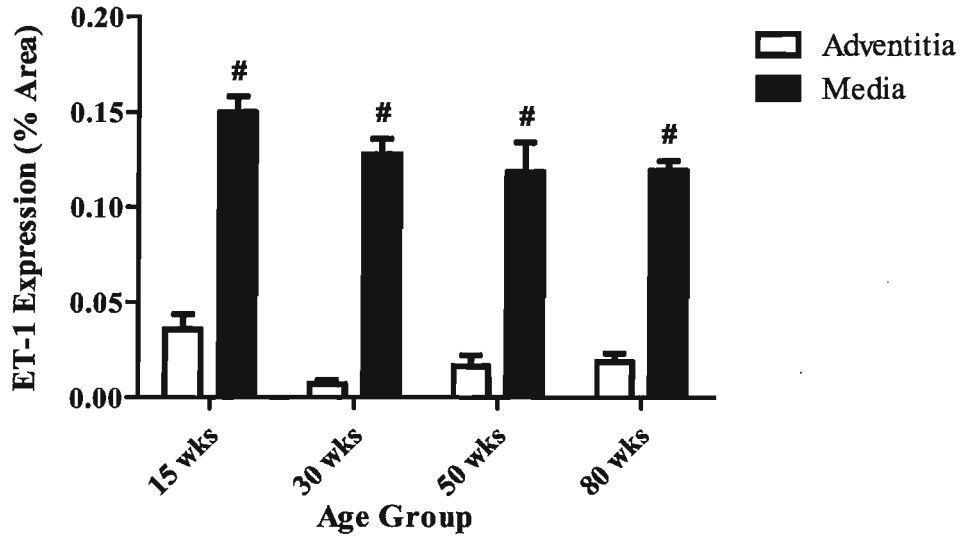

Figure 13. Quantitative analysis of ET-1 protein expression in the aortic wall. ET-1 protein was expressed consistently in the medial layer (filled bars) at all ages, yet showed an inconsistent expression pattern in the adventitia (open bars). In the adventitia ET-1 expression was significantly lower in the 30 and 80 week old groups compared to the 15 week old controls. ET-1 expression was significantly greater in the medial layer compared to the adventitial layer at all time points. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. *$p<0.05$ vs. 15wks group, #$p<0.05$ vs. corresponding adventitia; N=4-5 per group.

4.3.1.2 ECE-1

The expression of ECE-1 in the aorta was localized to a thin band on the endothelium (present in some sections), and as a thick swath through the adventitia (Figure 14).

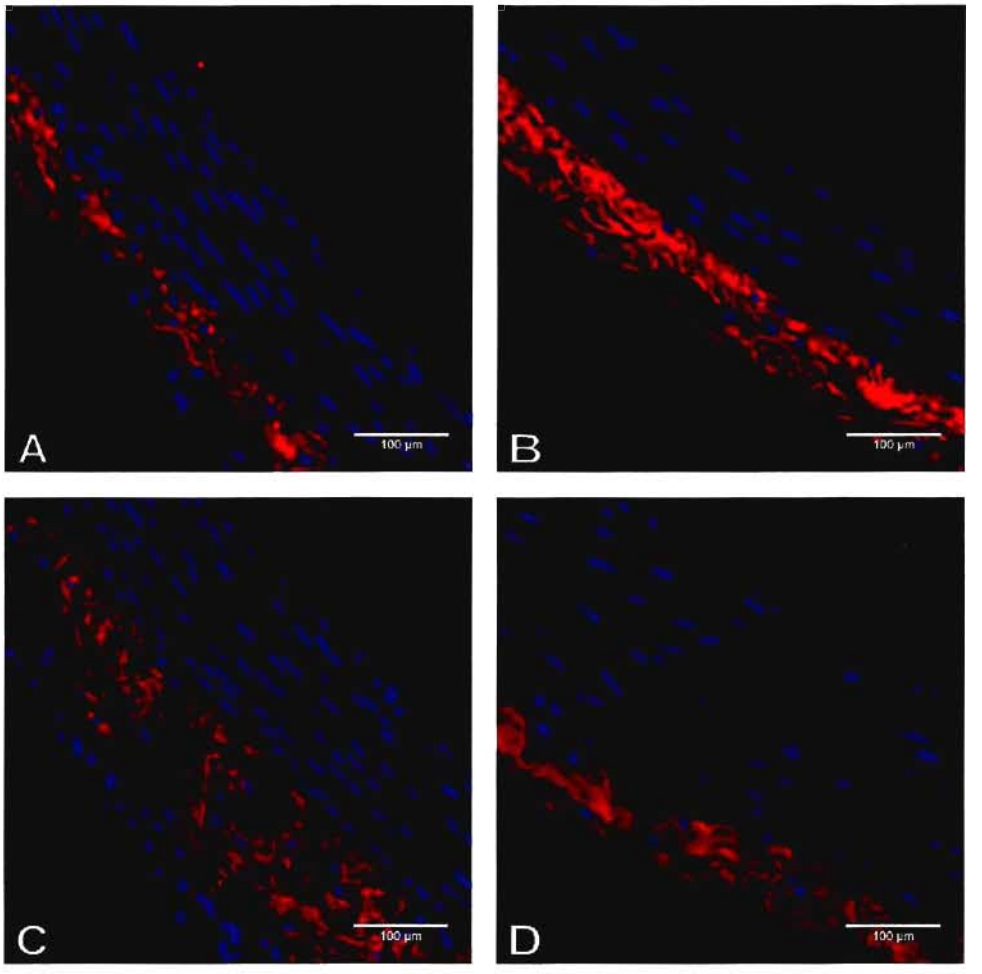

Figure 14. ECE-1 protein localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C) and 80 weeks (D) all expressed ECE-1 abundantly in the vascular adventitia. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for ECE-1 and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Quantitative analysis of ECE-1 expression in the aorta revealed consistent expression across all age groups in both the adventitial and medial layers (Figure 15). However, expression of ECE-1 was on the order of 10 to 30-fold greater in the adventitia

than in the media at all time points (Figure 15).

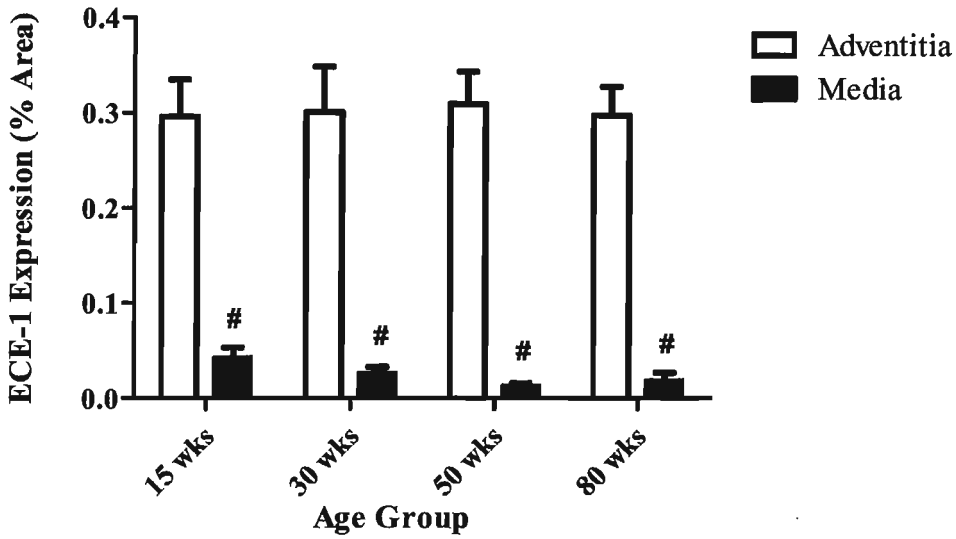

Figure 15. Quantitative analysis of ECE-1 protein expression in the aortic wall. ECE-1 protein was expressed robustly and consistently in the adventitial layer (open bars) at all ages, yet showed minimal expression in the media (filled bars). Expression was significantly greater in the adventitia than the media at all time points. However, expression did not differ with increasing age in neither the adventitia nor the media. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. #$p<0.05$ vs. corresponding adventitia; N=6-7 per group.

4.3.1.3 ET_A Receptor

The expression of the ET_A receptor in the aorta exhibited a similar pattern to that of the ET-1 peptide. Expression was not localized to any layer or compartment, but expressed throughout the vessel wall (Figure 16).

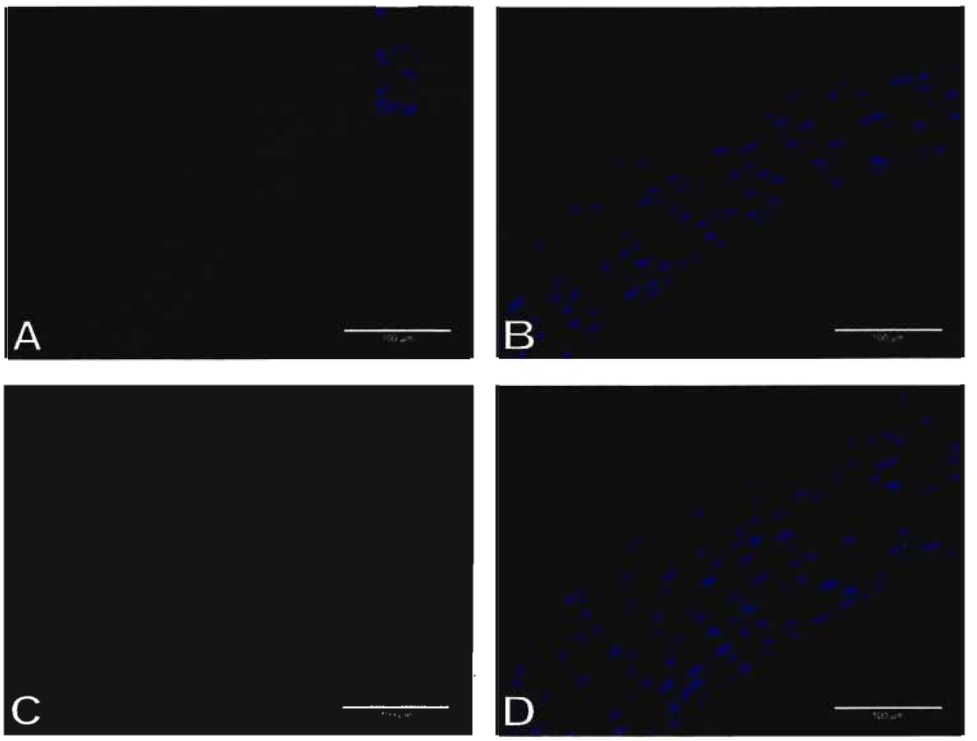

Figure 16. ET_A receptor protein localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) expressed the ET_A receptor throughout the vessel wall. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5μm. Samples were then immunostained for ET_A receptor and detected with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Zeiss fluorescent microscope with a 20X objective lens. Scale bars represent 100μm.

A closer quantitative examination of ET_A receptor expression in the aorta revealed a consistently greater expression in the medial layer, as compared to the adventitia, at all time points (Figure 17). Expression of the ET_A receptor in the adventitia did not vary across the different age groups, but in the media was greater, although not significantly, in the 80 week old group as compared to all other time points (Figure 17).

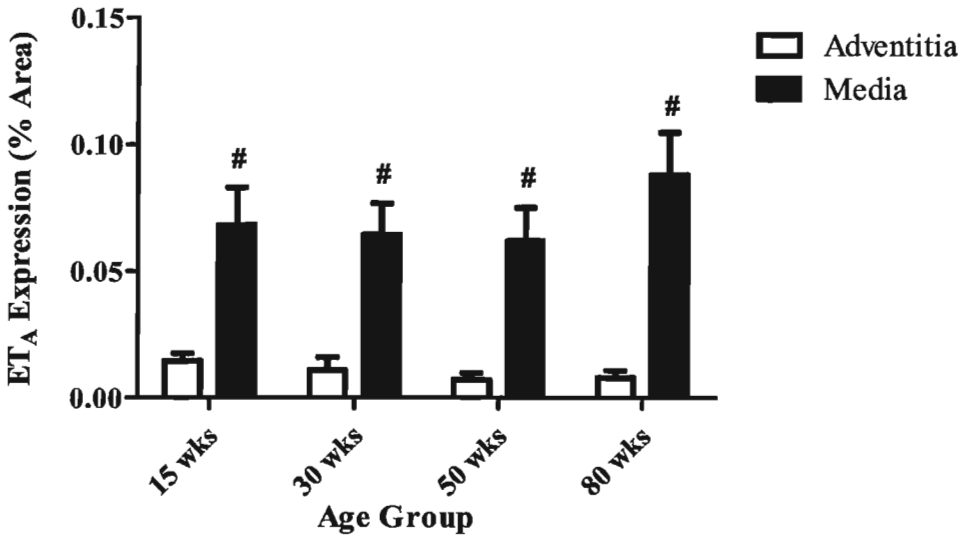

Figure 17. Quantitative analysis of ET_A receptor protein in the aortic wall. ET_A receptor expression did not change significantly with age in neither the adventitia (open bars) nor the media (filled bars). However, ET_A receptor expression was significantly greater in the medial layer as compared to the adventitial layer at all time points. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. #$p<0.05$ vs. corresponding adventitia; N=5-6 per group.

4.3.1.4 ET_B Receptor

The expression of the ET_B receptor in the aorta followed a similar pattern to that of ECE-1 expression, exhibiting a thin band on the endothelium and a thick swath in the adventitia (Figure 18).

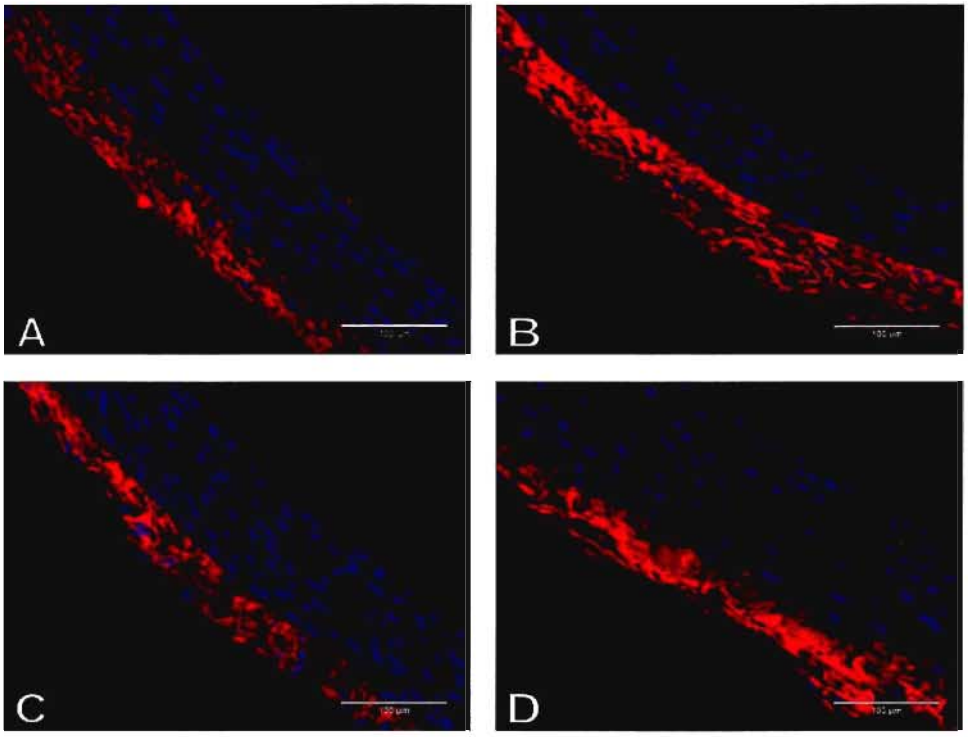

Figure 18. ET_B receptor protein localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) expressed the ET_B receptor abundantly in the adventitia. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5μm. Samples were then immunostained for ET_B receptor and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Zeiss fluorescent microscope with a 20X objective lens. Scale bars represent 100μm.

As with ECE-1 expression, the expression of the ET_B receptor in the adventitia and media was consistent across all age groups. However, the adventitia exhibited 30 to 80-fold greater ET_B receptor protein expression than that of the medial layer (Figure 19).

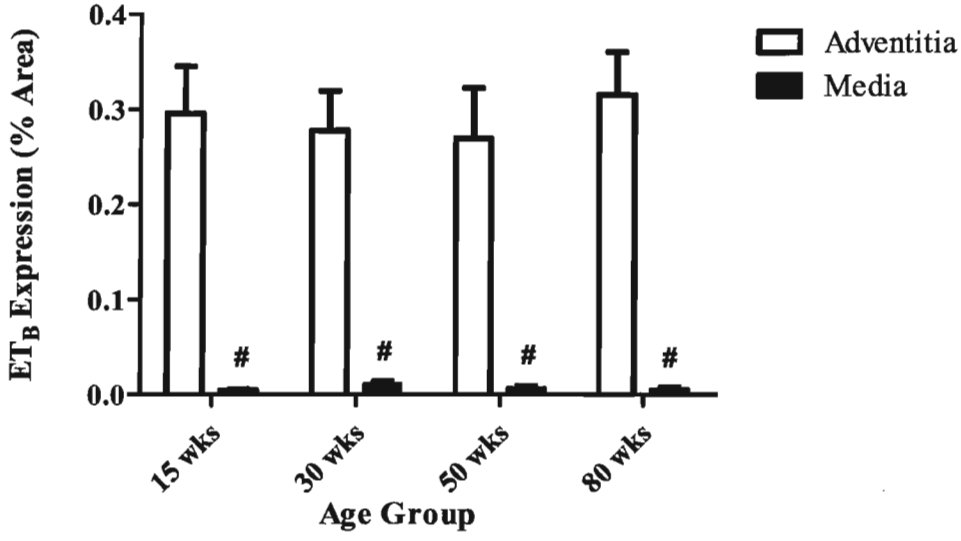

Figure 19. Quantitative analysis of ET_B receptor protein expression in the aortic wall. The ET_B receptor was expressed robustly and consistently in the adventitial layer (open bars), but displayed significantly less expression in the medial layer (filled bars) of all age groups. ET_B receptor expression did not differ in neither the adventitial nor the medial layers with increasing age. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. #$p<0.05$ vs. corresponding adventitia; N=4-5 per group.

4.3.2 Mesenteric Arteries

4.3.2.1 ET-1

As with ET-1 expression in the aorta, expression of ET-1 in the mesenteric arteries did not appear to localize to any layer, with expression spread throughout the vessel wall (Figure 20).

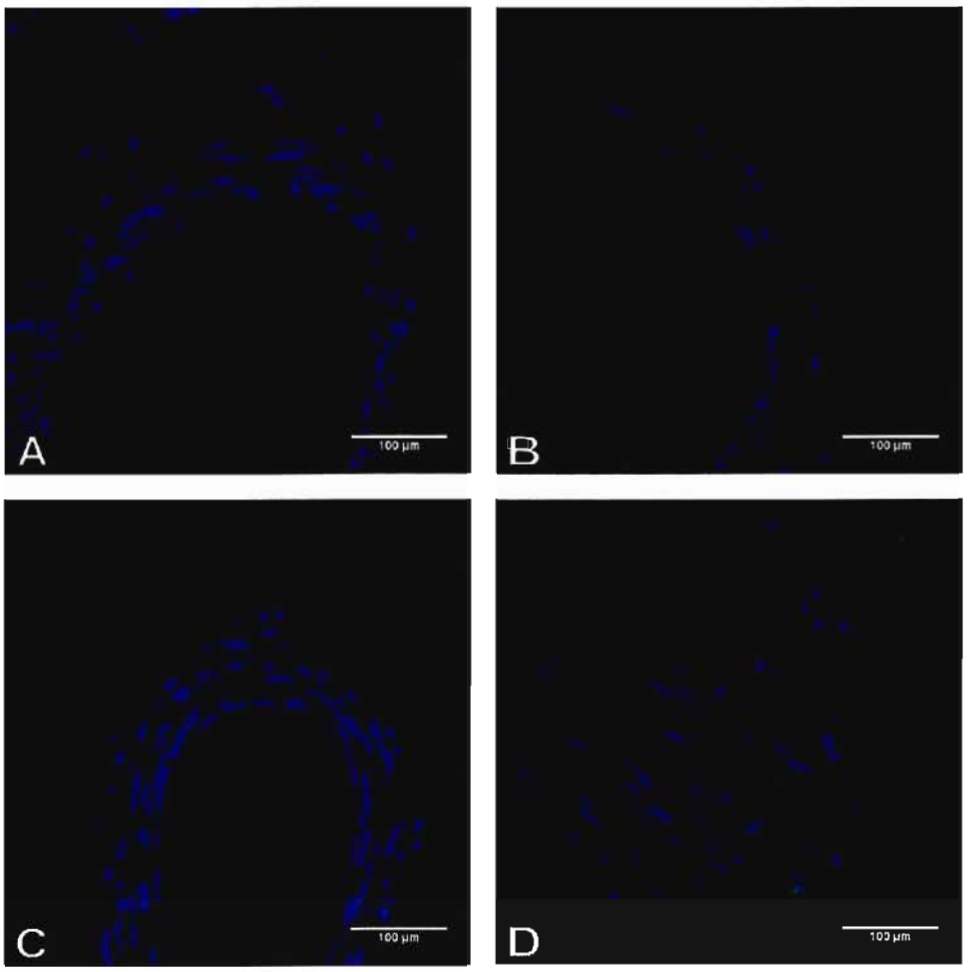

Figure 20. ET-1 protein localization in the mesenteric artery wall. Rat mesenteric arteries aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) displayed ET-1 protein localized throughout the mesenteric artery wall, with slightly more in the medial layer. Mesenteric arteries were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for ET-1 and detected with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Quantitative analysis of ET-1 expression in the mesenteric arteries revealed no significant changes in expression across time points in either of the adventitial or medial layers (Figure 20). With the exception of 15 weeks of age, ET-1 expression in the medial

layer was greater than the adventitial layer, reaching significance at 30 weeks of age (Figure 21).

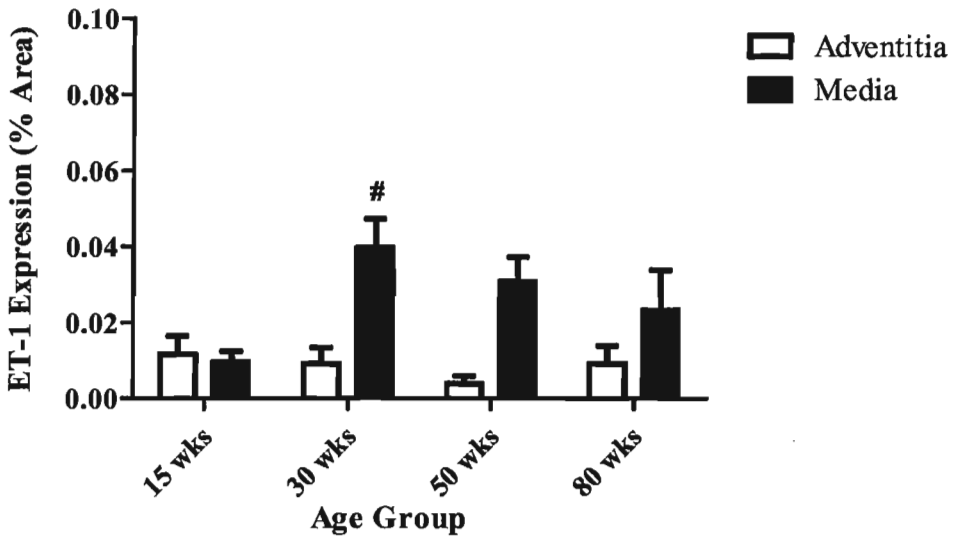

Figure 21. Quantitative analysis of ET-1 protein expression in the mesenteric artery wall. ET-1 protein expression did not differ significantly across time points in either the adventitia (open bars) or the media (filled bars). However, ET-1 protein expression was significantly greater in the medial layer at 30 weeks of age as compared to the adventitia. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. #$p<0.05$ vs. corresponding adventitia; N=3-4 per group.

4.3.2.2 ECE-1

Expression of ECE-1 in the mesenteric arteries followed a similar pattern to that of the aorta. Expression was localized to a thin band on the endothelium, as well as a thick swath in the adventitia (Figure 22).

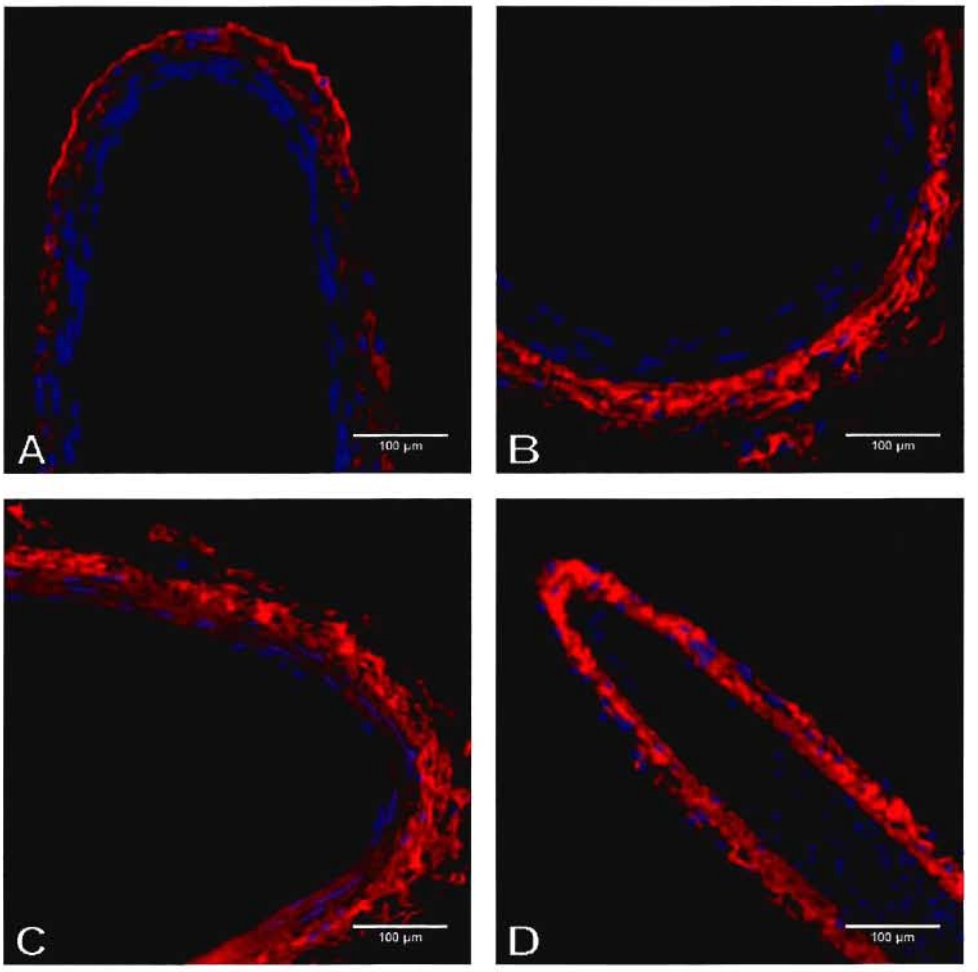

Figure 22. ECE-1 protein localization in the mesenteric artery wall. Rat mesenteric arteries aged 15 weeks (A), 30 weeks (B), 50 weeks (C) and 80 weeks (D) all expressed ECE-1 abundantly in the vascular adventitia. Mesenteric arteries were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for ECE-1 and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Quantification of ECE-1 expression in the mesenteric arteries revealed fairly consistent expression levels from 15 to 50 weeks in the adventitial layer, with a sharp significant increase at 80 weeks of age (Figure 23). On the other hand, medial expression

of ECE-1 remained consistent across all time points. At 15, 30 and 80 weeks of age, expression of ECE-1 in the mesenteric artery adventitia was significantly higher than expression in the corresponding medial layer (Figure 23).

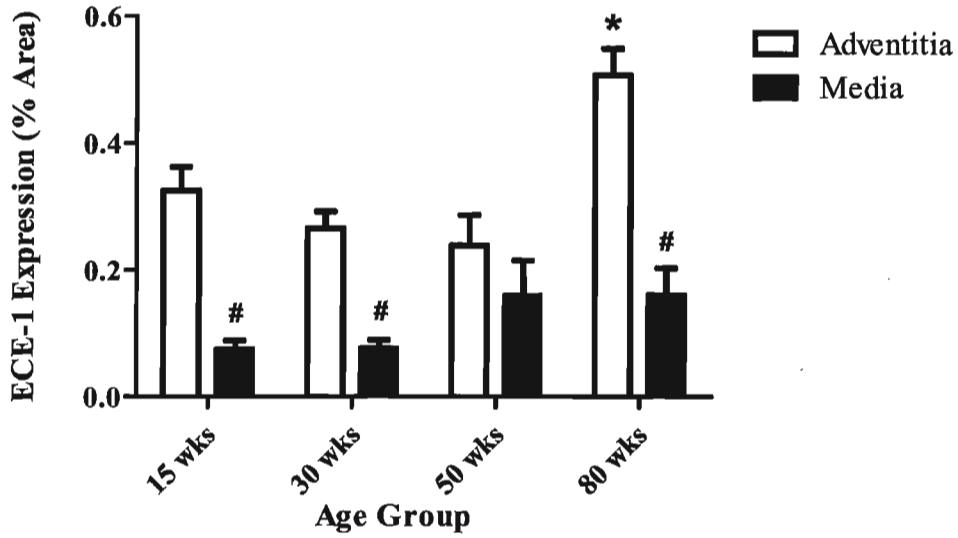

Figure 23. Quantitative analysis of ECE-1 protein expression in the mesenteric artery wall. ECE-1 protein expression did not differ significantly across time points in the medial layer (filled bars). However, ECE-1 protein expression was significantly greater in at 80 weeks of age as compared to all other time points in the adventitia (open bars). At 15, 30, and 80 weeks of age, ECE-1 expression was significantly greater in the adventitia as compared to the medial layer. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. *$p<0.05$ vs. 15wks group, #$p<0.05$ vs. corresponding adventitia; N=6-7 per group.

4.3.2.3 ET_A Receptor

As with the aorta, expression of the ET_A receptor did not appear to localize to any specific layer (Figure 24).

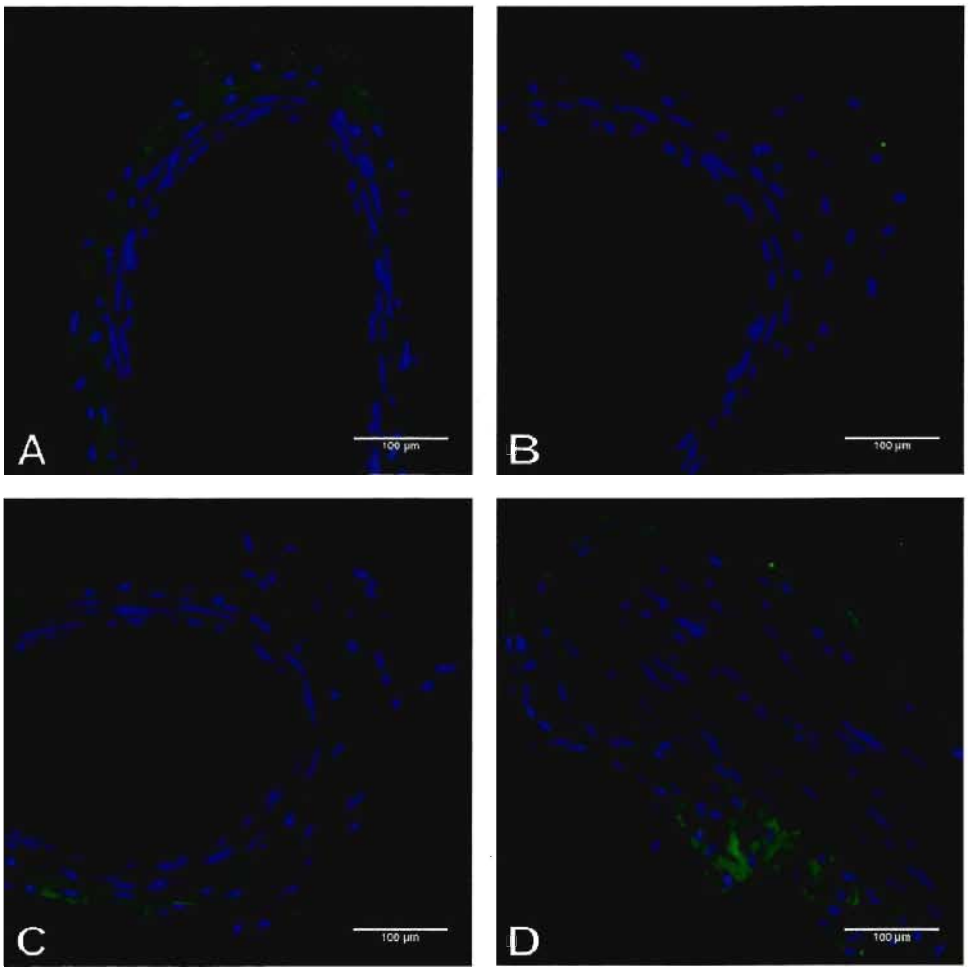

Figure 24. ET_A receptor protein localization in the mesenteric artery wall. Rat mesenteric arteries aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) displayed ET_A receptor protein localized throughout the mesenteric artery wall. Mesenteric arteries were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for ET_A receptor and detected with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Further quantitative analysis of ET_A receptor expression in the mesenteric arteries revealed no significant changes in expression across time points in the adventitial layer (Figure 25). In the media, expression of the ET_A receptor was significantly higher at 80

weeks of age compared to the 15 week old controls (Figure 25). Additionally, no significant differences were observed between the adventitia and media at all time points (Figure 25).

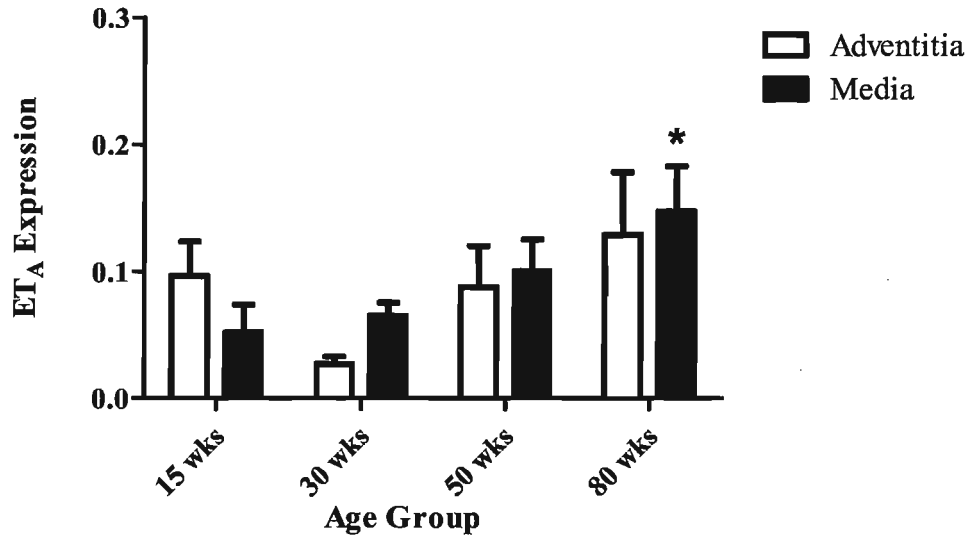

Figure 25. Quantitative analysis of ET_A receptor protein expression in the mesenteric artery wall. Expression of the ET_A receptor did not differ significantly across time points in the adventitia (open bars) but was significantly higher at 80 weeks as compared to 15 weeks in the media (filled bars). Additionally, no difference in expression was observed between the adventitial and medial layers at any time point. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. *$p<0.05$ vs. corresponding adventitia; N=5 per group.

4.3.2.4 ET_B Receptor

As in the aortic sections, expression of the ET_B receptor protein was highly localized to the adventitial layer of the mesenteric arteries, along with a thin band in the endothelium (Figure 26).

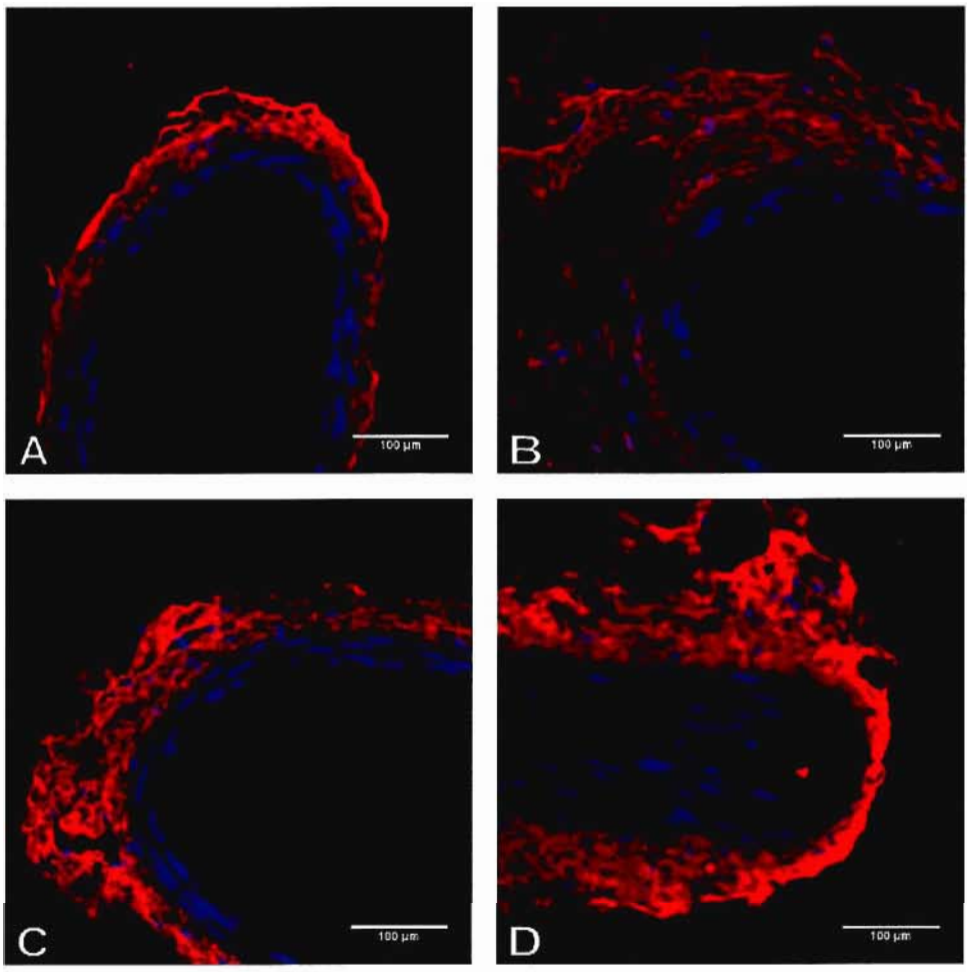

Figure 26. ET_B receptor protein localization in the mesenteric artery wall. Rat mesenteric arteries aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) expressed the ET_B receptor abundantly in the adventitia. Mesenteric arteries were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for ET_B receptor and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

A closer quantitative examination of the ET_B receptor protein expression in the mesenteric arteries revealed a consistent expression level in the adventitia from 15 to 50 weeks of age, with a large significant increase at 80 weeks of age (Figure 27). ET_B

receptor protein expression was fairly consistent across time points in the medial layer (Figure 27). At all time points, ET_B receptor protein expression was significantly higher in the adventitia than in the media (Figure 27).

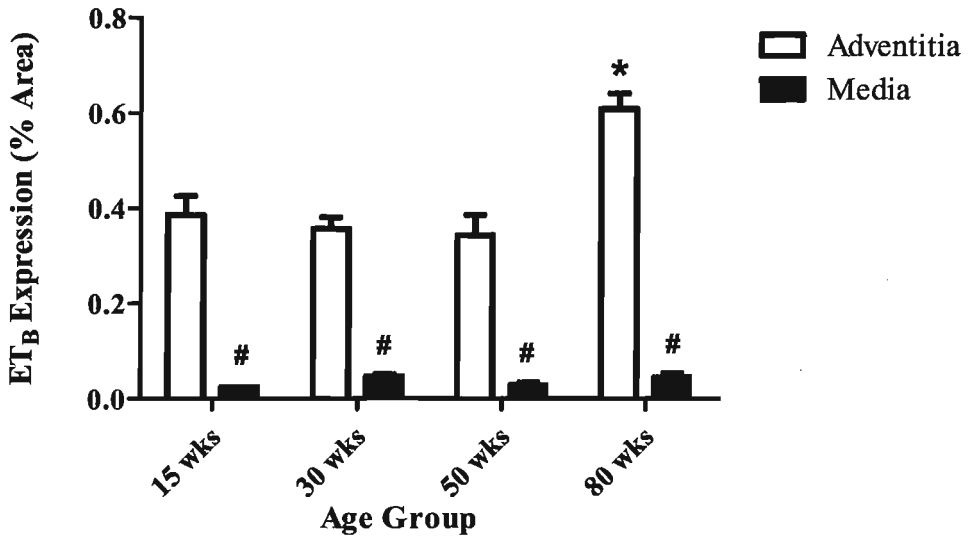

Figure 27. Quantitative analysis of ET_B receptor protein expression in the mesenteric artery wall. In the adventitia (open bars), expression of the ET_B receptor was significantly greater at 80 weeks of age compared to all other time points. Meanwhile, in the media (filled bars), ET_B receptor expression did not differ across time points. At all time points, ET_B receptor expression was significantly greater in the adventitia as compared to the media. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. *$p<0.05$ vs. 15wks group, #$p<0.05$ vs. corresponding adventitia; N=5 per group.

4.3.3 Pudendal Arteries

4.3.3.1 ET-1

As with the aortic and mesenteric artery sections, ET-1 expression did not appear

to localize to any specific layer in the pudendal arteries (Figure 28).

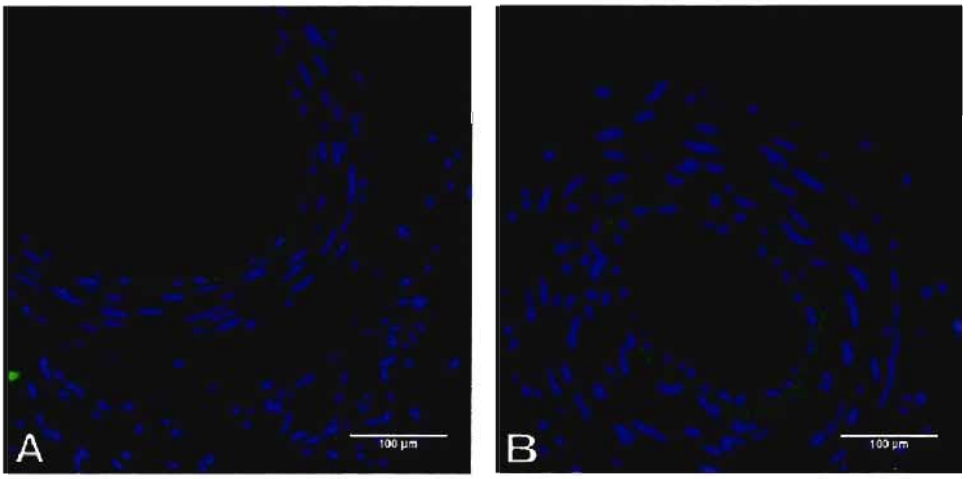

Figure 28. ET-1 protein localization in the pudendal artery wall. Rat pudendal arteries aged 15 weeks (A) and 80 weeks (B) displayed ET-1 protein localized to the entire pudendal artery wall, with a sharp band at the endothelium. Pudendal arteries were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for ET-1 and detected with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Quantification of ET-1 expression in the pudendal arteries revealed a sharp drop in expression with increasing age in both the adventitial and medial layers (Figure 29). Furthermore, the expression of ET-1 did not differ significantly between the adventitia and media at either time point (Figure 29).

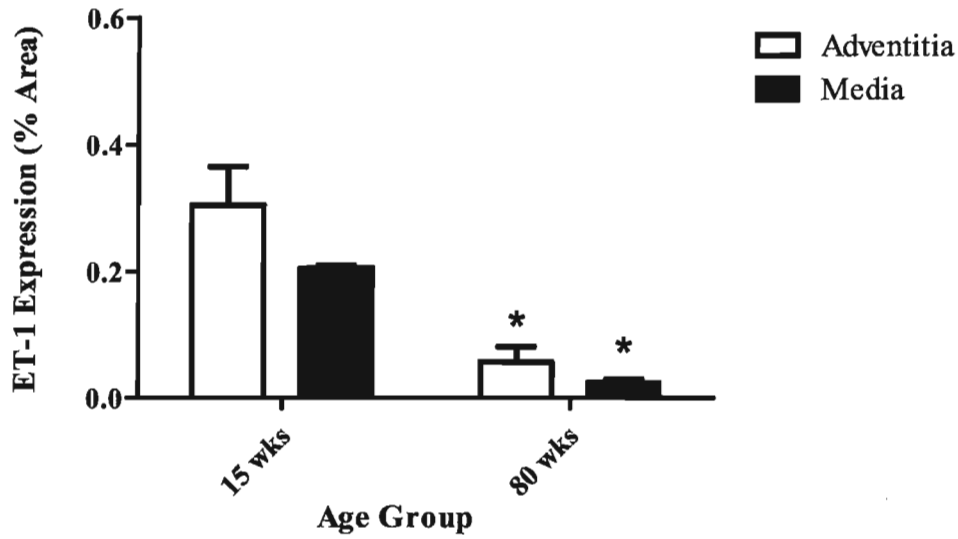

Figure 29. Quantitative analysis of ET-1 protein expression in the pudendal artery wall. ET-1 protein expression in the pudendal arteries was greater in the 15 week old group than in the 80 week old group in both the adventitia (open bars) and the media (filled bars). Furthermore ET-1 protein expression did not differ between the adventitia and media at each time point. Data are expressed as mean ± SEM. Data were analyzed by Student's t-test. *$p<0.05$ vs. 15wks group; N=4-5 per group.

4.3.3.2 ECE-1

Similarly to the aortic and mesenteric artery sections, the pudendal arteries displayed a high degree of localization of ECE-1 protein in the adventitial layer, with much less in the medial layer (Figure 30).

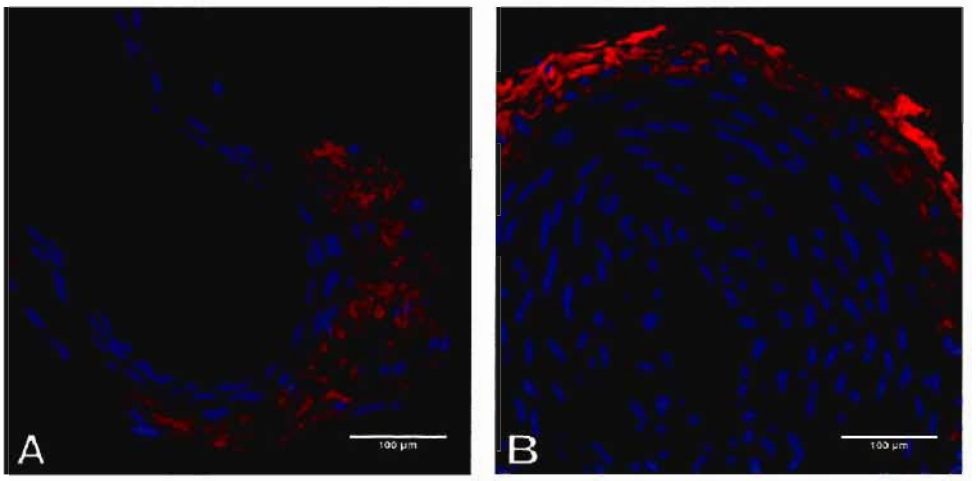

Figure 30. ECE-1 protein localization in the pudendal artery wall. Rat pudendal arteries aged 15 weeks (A) and 80 weeks (B) displayed ECE-1 protein localized primarily to the adventitial layer. Pudendal arteries were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for ECE-1 and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Quantitative analysis of ECE-1 protein expression in the pudendal arteries revealed a large significant increase in expression with increasing age in the adventitial layer, but a non-significant decrease in expression in the medial layer (Figure 31). At each time point, the level of ECE-1 expression was greater in the adventitia than in the media (Figure 31).

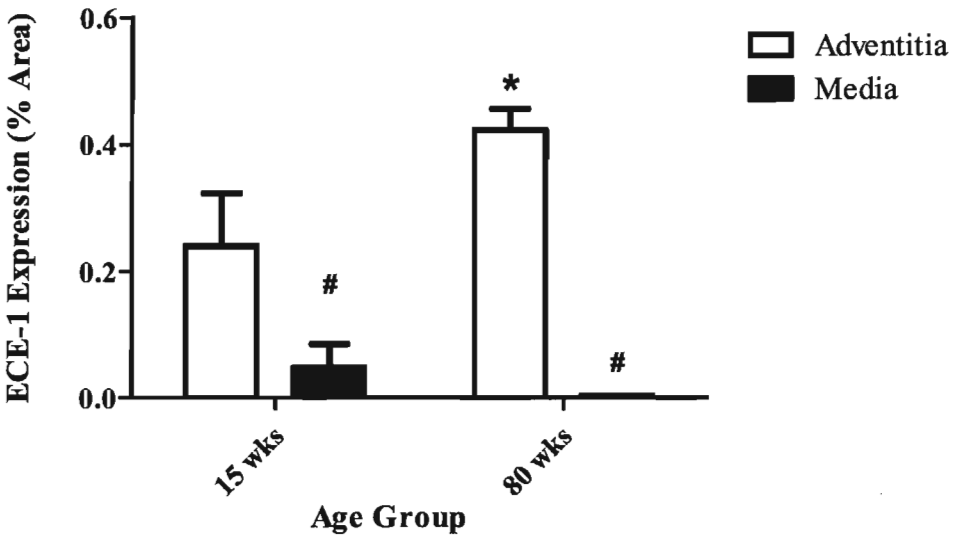

Figure 31. Quantitative analysis of ECE-1 protein expression in the pudendal artery wall. ECE-1 protein expression did not differ significantly across time points in the medial layer (filled bars). However, ECE-1 protein expression was significantly greater in at 80 weeks of age as compared to 15 weeks in the adventitia (open bars). At both time points, ECE-1 expression was significantly greater in the adventitia as compared to the medial layer. Data are expressed as mean ± SEM. Data were analyzed by Student's t-test. *$p<0.05$ vs. 15wks group, #$p<0.05$ vs. corresponding adventitia; N=5-6 per group.

4.3.3.3 ET_A receptor

Expression of the ET_A receptor in the pudendal arteries followed a similar pattern as exhibited in the aortic and mesenteric artery sections, displaying no apparent localization to any specific layer (Figure 32).

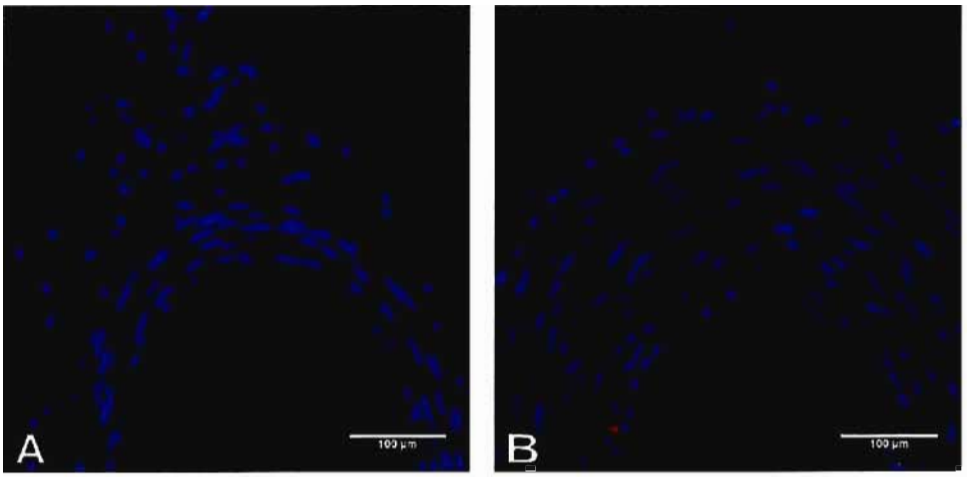

Figure 32. ET_A receptor protein localization in the pudendal artery wall. Rat pudendal arteries aged 15 weeks (A) and 80 weeks (B) displayed ET_A receptor protein localized throughout the mesenteric artery wall. Pudendal arteries were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for ET_A receptor and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Further quantitative analysis of ET_A receptor protein expression in the pudendal arteries revealed a large significant decline in expression with increasing age in the adventitial layer while exhibiting no change in the medial layer (Figure 33). At 15 weeks of age, ET_A receptor expression was significantly greater in the adventitia compared to the media, but no such difference existed at 80 weeks (Figure 33).

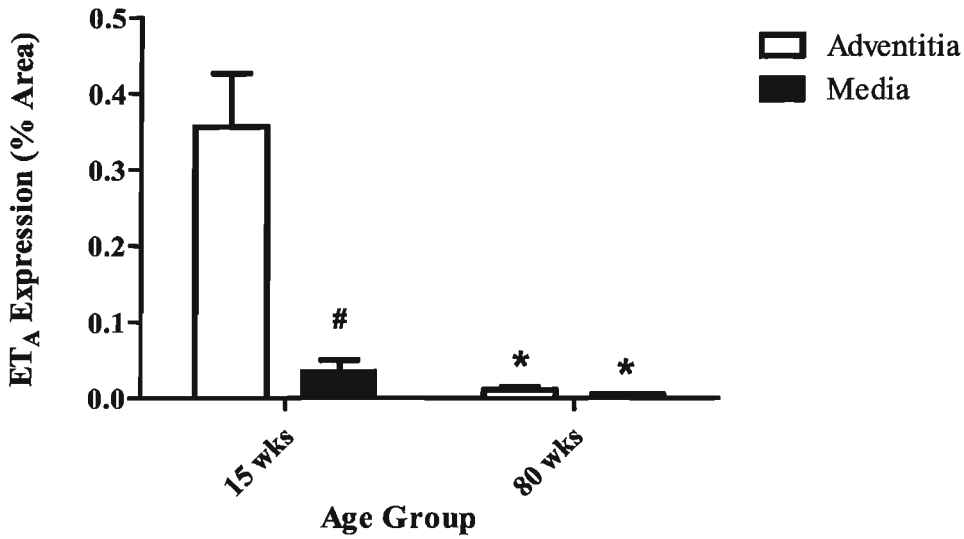

Figure 33. Quantitative analysis of ET_A receptor protein expression in the pudendal artery wall. ET_A receptor expression was significantly lower at 80 weeks of age as compared to 15 weeks in the adventitia (open bars) and the media (filled bars). ET_A receptor expression was significantly greater in the adventitia compared to the media at 15 weeks, but did not differ significantly at 80 weeks. Data are expressed as mean ± SEM. Data were analyzed by Student's t-test. *$p<0.05$ vs. 15wks group, #$p<0.05$ vs. corresponding adventitia; N=5 per group.

4.3.3.4 ET_B receptor

Expression of ET_B receptor protein in the pudendal artery wall followed a similar pattern as in the aorta and mesenteric arteries, with a high degree of localization in the adventitial layer (Figure 34).

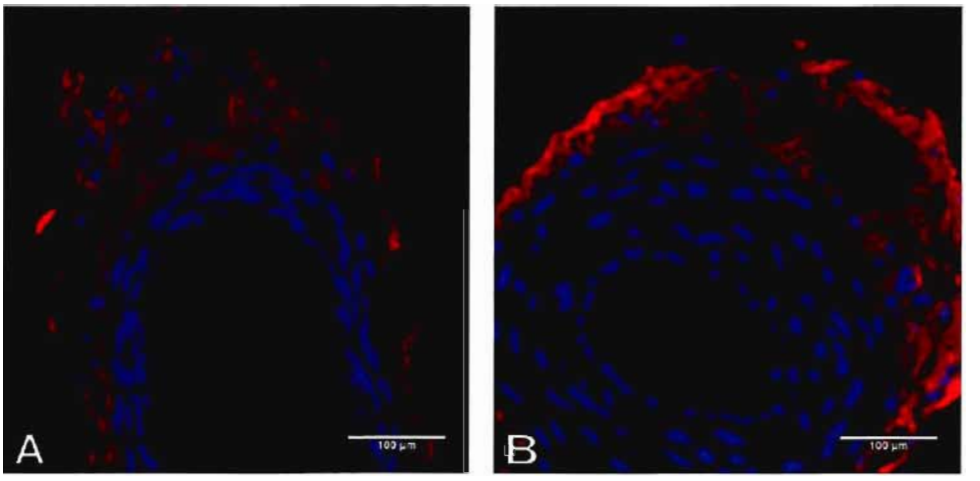

Figure 34. ET_B receptor protein expression in the pudendal artery wall. Rat pudendal arteries aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) expressed the ET_B receptor abundantly in the adventitia. Pudendal arteries were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5μm. Samples were then immunostained for ET_B receptor and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100μm.

Quantitative analysis of ET_B receptor protein expression revealed a small significant rise in expression in the adventitial layer from 15 to 80 weeks (Figure 35). Contrarily, expression in the medial layer exhibited a small significant decline from 15 to 80 weeks (Figure 35). At each time point, expression of ET_B receptor protein was higher in the adventitial layer than in the corresponding medial layer (Figure 35).

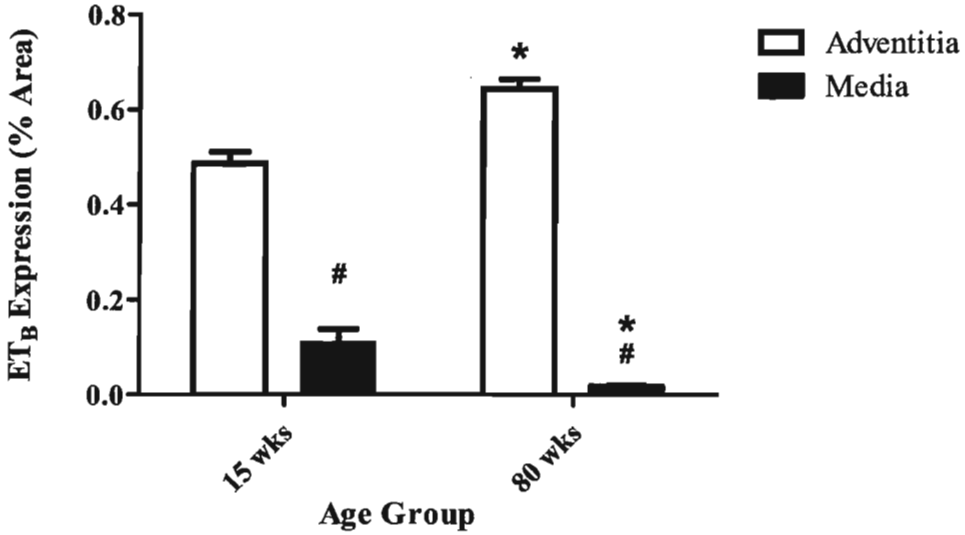

Figure 35. Quantitative analysis of ET_B receptor protein expression in the pudendal artery wall. ET_B receptor expression was significantly greater at 80 weeks of age as compared to 15 weeks in the adventitia (open bars) but was significantly lower at 80 weeks of as compared to 15 weeks of age in the media (filled bars). ET_B receptor expression was significantly greater in the adventitia than in the media at each time point. Data are expressed as mean ± SEM. Data were analyzed by Student's t-test. *$p<0.05$ vs. 15wks group, #$p<0.05$ vs. corresponding adventitia; N=6 per group.

4.4 Oxidative Damage, Extracellular Matrix and Myofibroblast Induction

4.4.1 Oxidative Damage

Expression of the oxidative stress marker, 3-nitrotyrosine, was not localized to any layer or compartment of the aortic wall, and was spread throughout the vessel wall (Figure 36).

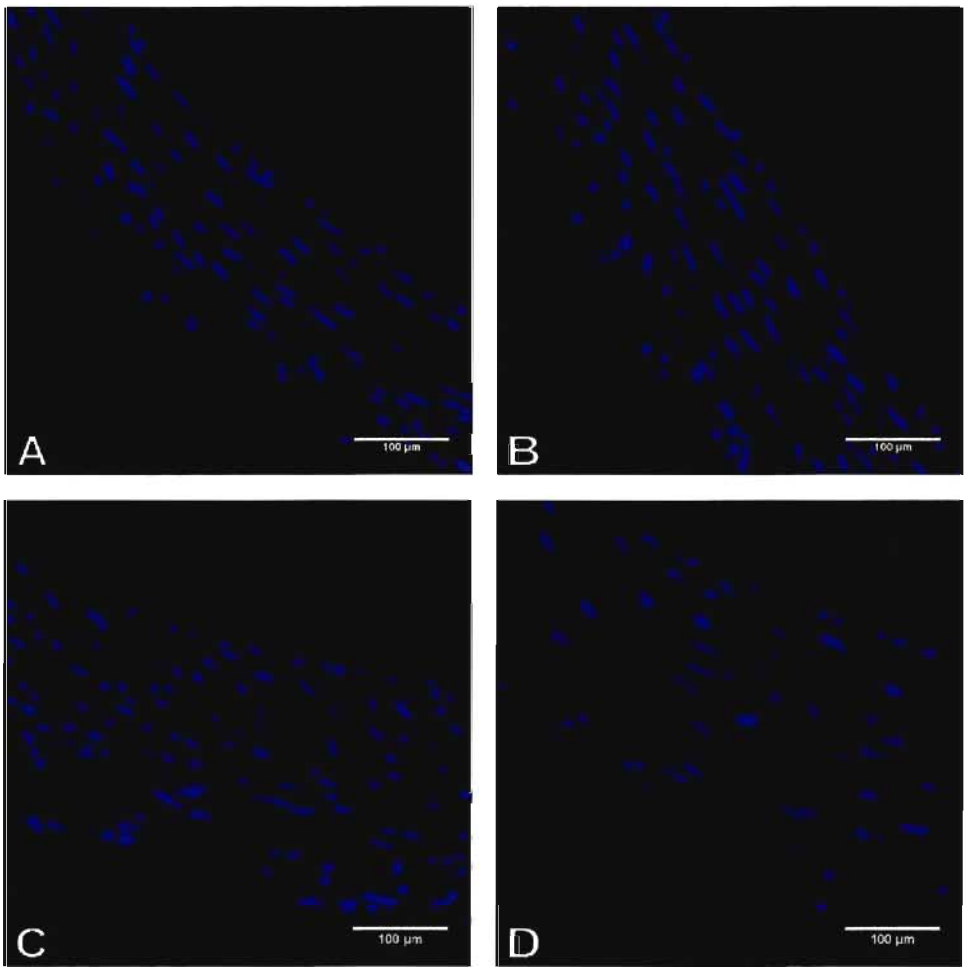

Figure 36. 3-Nitrotyrosine localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) exhibited 3-nitrotyrosine throughout the vessel wall. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for 3-nitrotyrosine and detected with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Further examination of 3-nitrotyrosine expression revealed somewhat higher expression in the medial layer however this was inconsistent across age groups. Expression was greatest in the adventitia at 15 and 50 weeks, but declined at 30 and 80

weeks, although not significantly (Figure 37). Expression of 3-nitrotyrosine in the medial layer remained consistent at 15, 30 and 50 weeks, but declined sharply at 80 weeks, although not significantly (Figure 37). Expression of 3-nitrotyrosine did not differ significantly between the adventitial and medial layers at all time points (Figure 37).

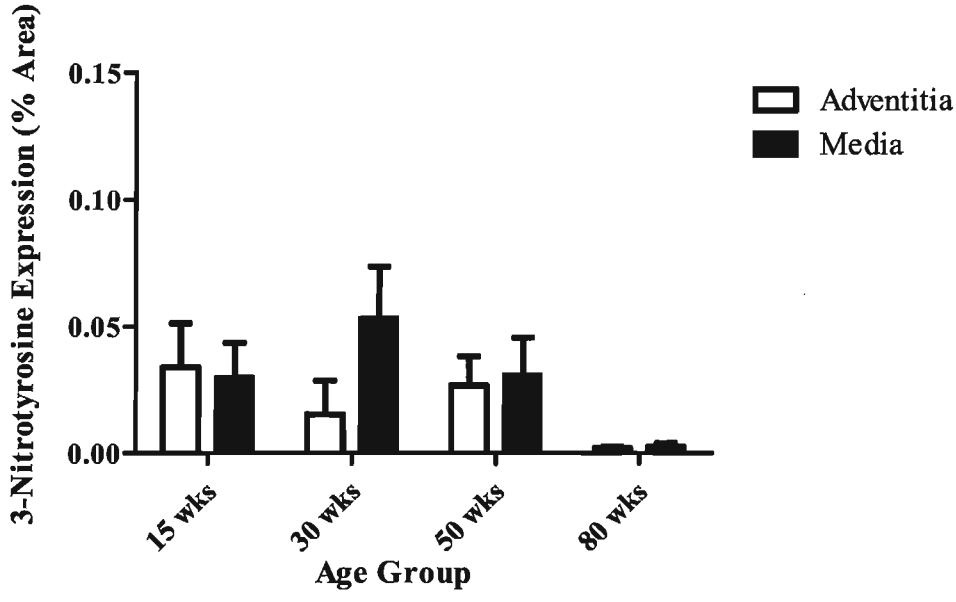

Figure 37. Quantitative analysis of 3-nitrotyrosine content in the aortic wall. 3-Nitrotyrosine displayed inconsistent expression in the adventitia (open bars) and the media (filled bars) with increasing age. Expression did not differ significantly between age groups in either layer. Similarly, expression did not differ significantly between layers at all time points. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. N=4 per group.

4.4.2 Extracellular Matrix Expression

4.4.2.1 Fibronectin

As expected the expression of fibronectin in the aorta was highly localized to the

adventitia, although expression was clearly present in the media (Figure 38).

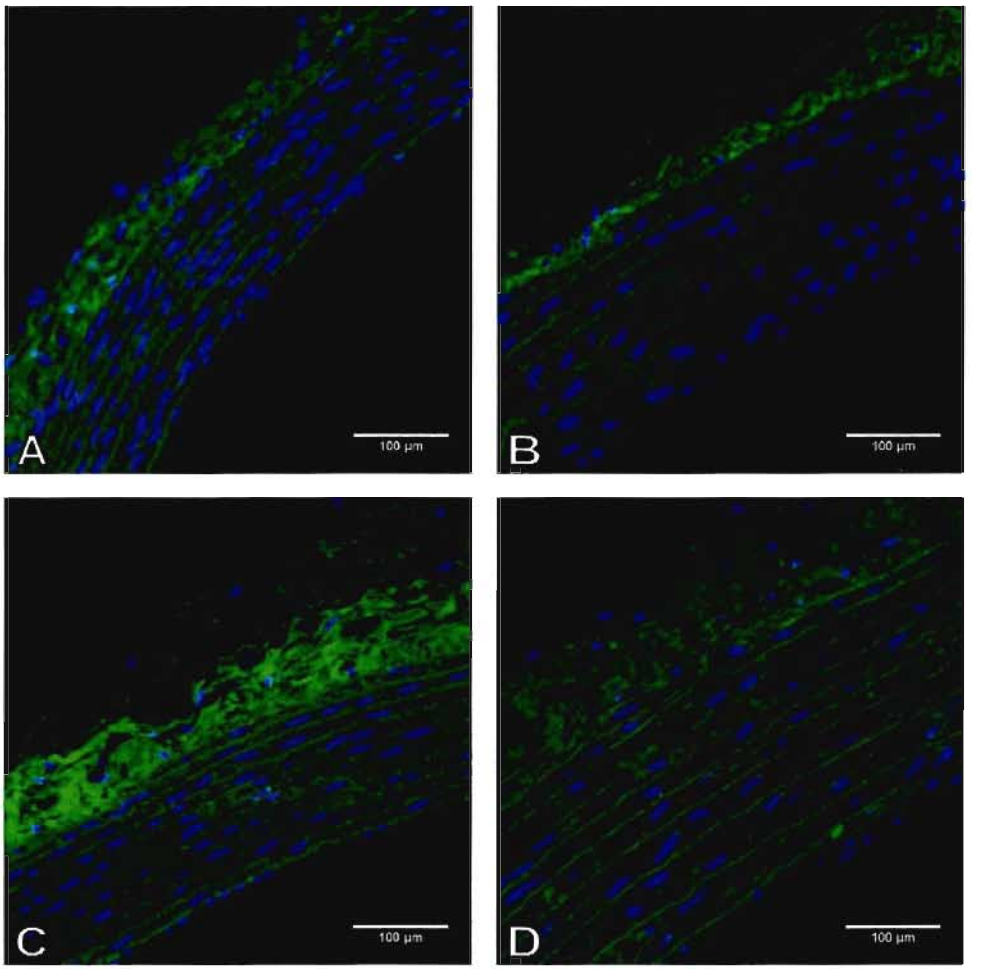

Figure 38. Fibronectin protein localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) expressed fibronectin abundantly in the adventitia, peaking at 50 weeks of age. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for fibronectin and detected with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Quantitative analysis of fibronectin expression in the aorta revealed consistent expression in the media across all age groups (Figure 39). On the other hand, expression

in the adventitia rose consistently from 15 through 50 weeks, before declining significantly at 80 weeks (Figure 39). At all time points, expression of fibronectin was greater in the adventitia than in the media, and was significantly so at 30 and 50 weeks of age (Figure 39).

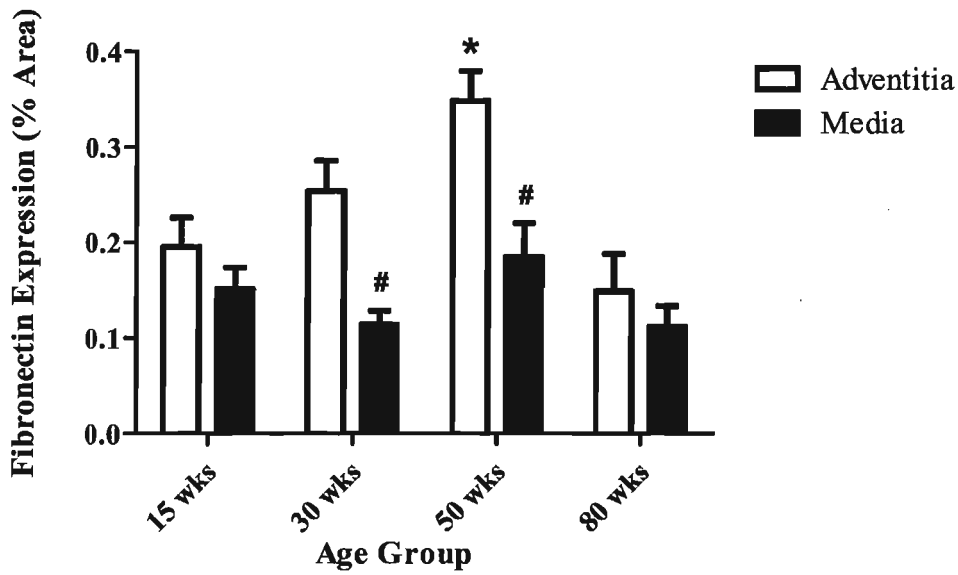

Figure 39. Quantitative analysis of fibronectin protein expression in the aortic wall. In the adventitia (open bars), fibronectin protein expression increased with age from 15 to 50 weeks of age, with significance being reached at 50 weeks compared to 15 weeks. Fibronectin protein expression did not differ significantly with increasing age in the medial layer (filled bars). Fibronectin expression was significantly greater in the adventitia compared to the media in the 30 and 50 week old groups ($p<0.05$). Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. *$p<0.05$ vs. 15wks groups, #$p<0.05$ vs. corresponding adventitia; N=5-7 per group.

Expression of fibronectin in the mesenteric arteries showed a similar pattern to that of the aorta, with a somewhat high degree of localization to the adventitia, but still displaying positive expression in the medial layer (Figure 40).

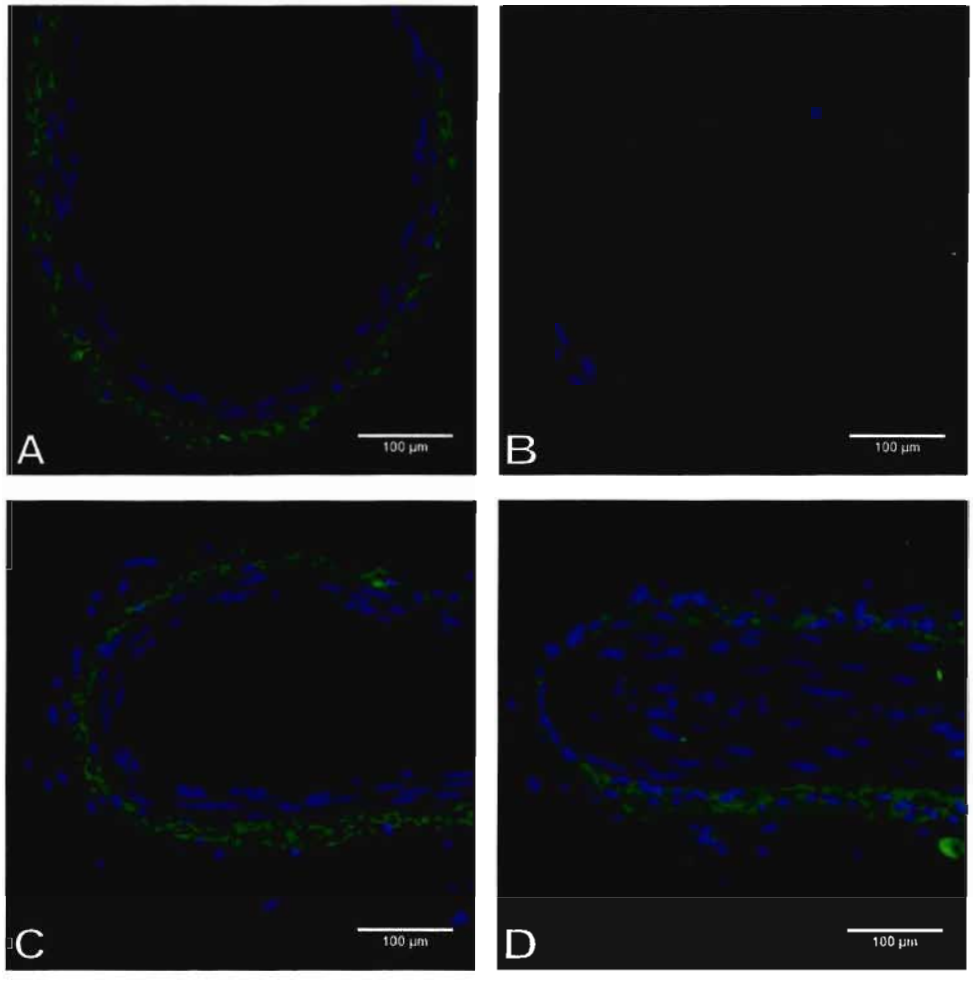

Figure 40. Fibronectin protein localization in the mesenteric artery wall. Rat mesenteric arteries aged 15 weeks (A), 30 weeks (B), 50 weeks (C) and 80 weeks (D) all expressed fibronectin abundantly in the vascular adventitia. Mesenteric arteries were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for fibronectin and detected with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

4.4.2.2 Collagen

Collagen type I expression in the aorta was localized primarily to the adventitia, with much less expression in the medial layer. Unlike fibronectin expression, collagen

type I was not expressed to a significant degree in the media (Figure 41).

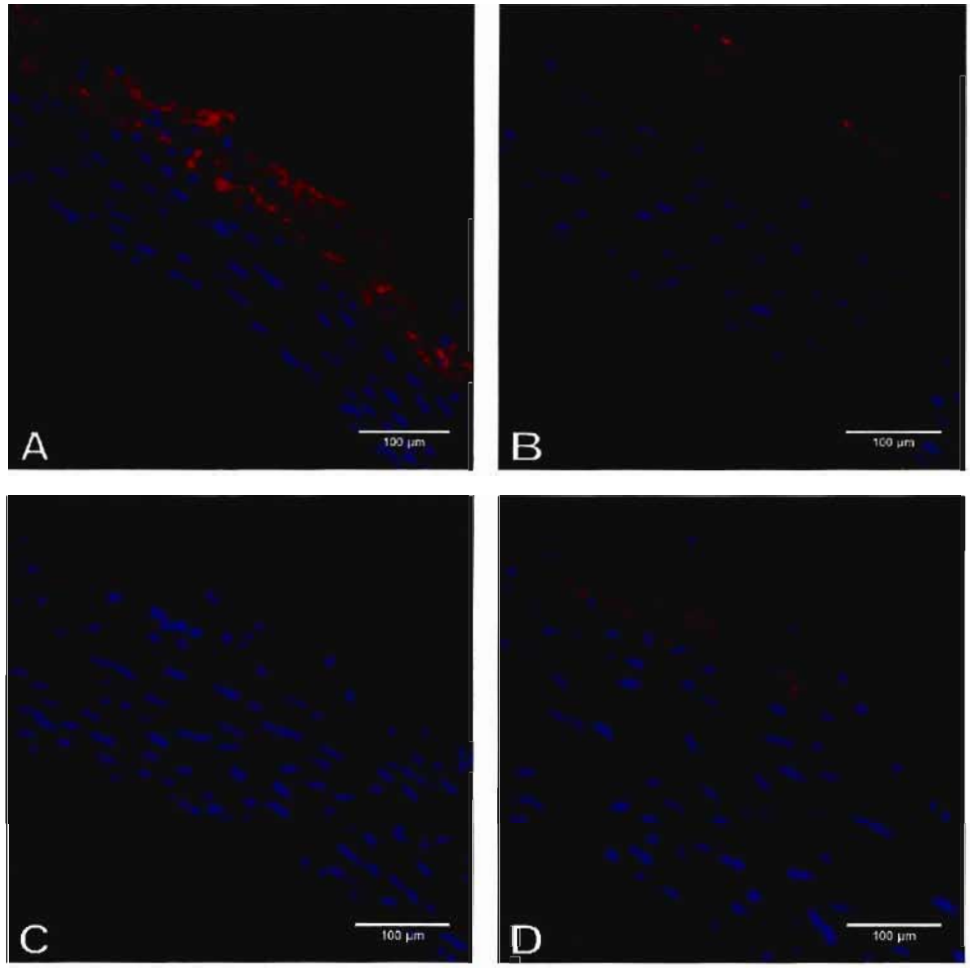

Figure 41. Collagen type I protein localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) expressed collagen type I abundantly in the adventitia. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for collagen type I and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Further examination showed higher collagen type I expression in the aortic adventitia at 15, 30 and 50 weeks, with less in the adventitia of the 30 week old aortas,

although this difference was not significant. Expression of collagen type I in the media was consistently low across all age groups (Figure 42). Expression of collagen type I was consistently higher in the adventitia than in the media at all time points, significantly so at 50 and 80 weeks of age (Figure 42).

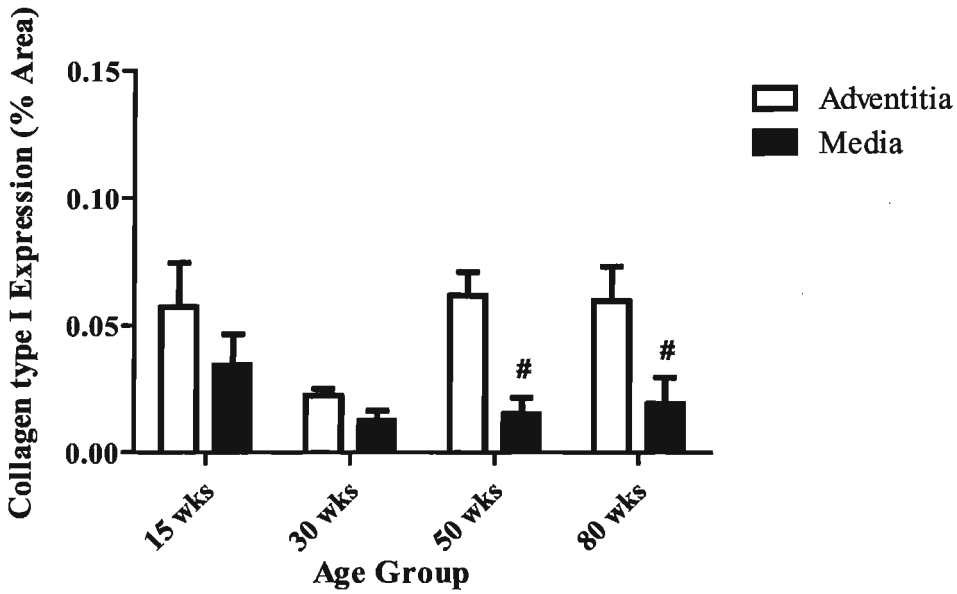

Figure 42. Quantitative analysis of collagen type I protein expression in the aortic wall. Collagen type I was expressed strongly in the adventitial layer (open bars) at 15, 50 and 80 weeks, but was somewhat weaker at 30 weeks, while expression in the media (filled bars) was low at all age groups. Furthermore, collagen type I expression was significantly higher in the adventitia compared to the media in the 50 and 80 week old groups. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. #$p<0.05$ vs. corresponding adventitia; N=4 per group.

As with the aorta, expression of collagen type I was greatest in the adventitia of the mesenteric arteries, and not expressed to a major degree in the medial layer (Figure 43).

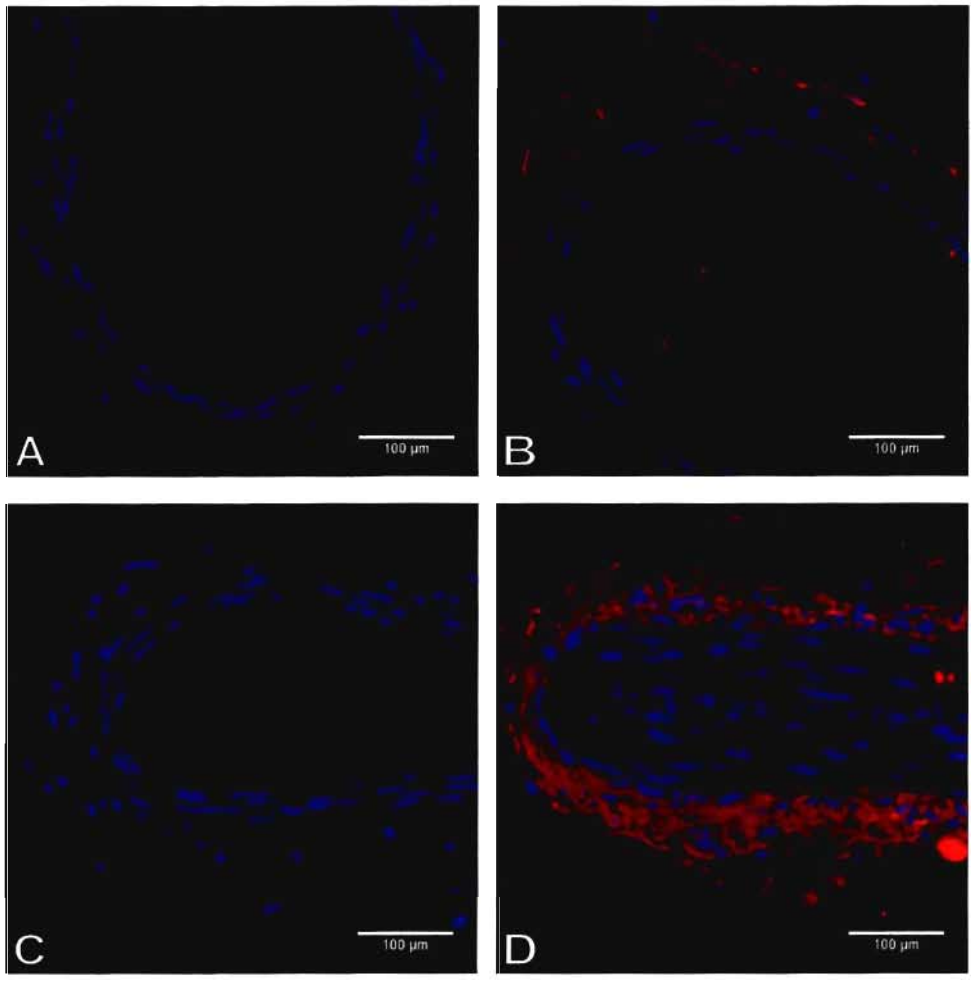

Figure 43. Collagen type I protein localization in the mesenteric artery wall. Rat mesenteric arteries aged 15 weeks (A), 30 weeks (B), 50 weeks (C) and 80 weeks (D) all expressed collagen type I abundantly in the vascular adventitia. Mesenteric arteries were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for collagen type I and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

4.4.3 Myofibroblast Induction

As expected α-SMA expression in the aortic wall was greatest in the medial layer,

with much less expression displayed in the adventitia (Figure 44).

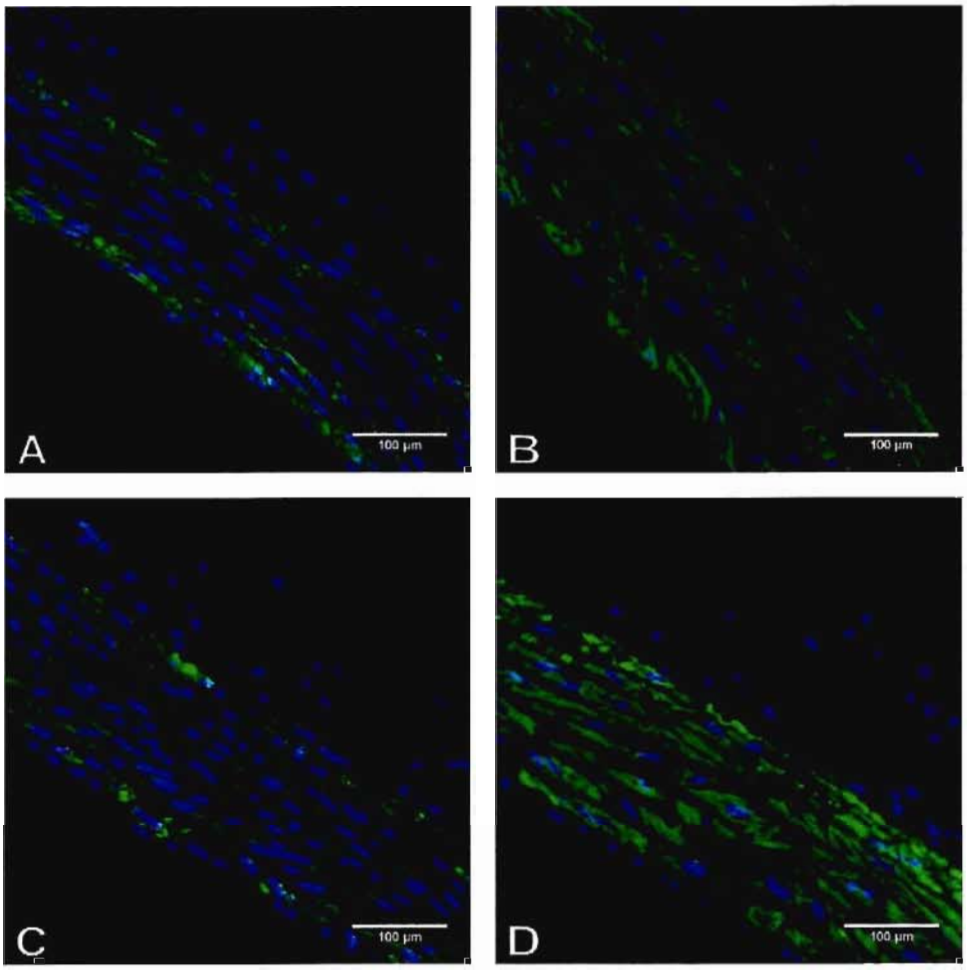

Figure 44. α-Smooth muscle actin protein localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) expressed α-smooth muscle actin primarily in the media, but only sporadically in the adventitia. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for α-smooth muscle actin and detected with a secondary antibody conjugated to Alexa Fluor 488 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

Further examination revealed consistently low expression of α-SMA in the aortic

adventitia across all age groups. However, expression in the medial layer remained relatively constant from 15 through 50 weeks, before increasing significantly at 80 weeks (Figure 45). Expression of α-SMA was consistently significantly higher in the medial layer than in the adventitia at all time points (Figure 45).

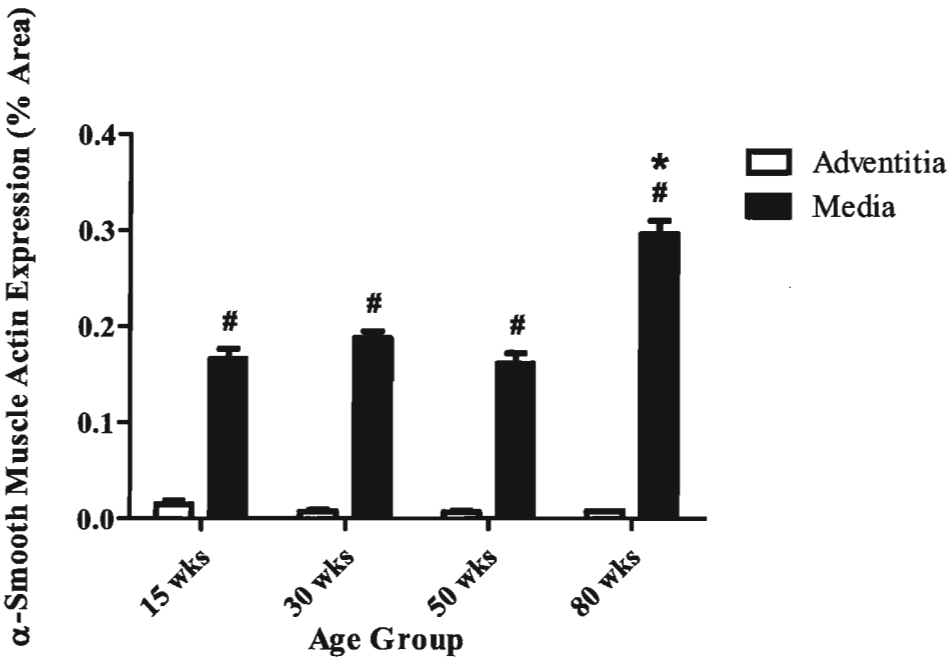

Figure 45. Quantitative analysis of α-smooth muscle actin protein expression in the aortic wall. α-Smooth muscle actin was expressed robustly in the medial layer (filled bars) at all age groups and displayed significantly greater expression at 80 weeks compared to all other age groups. Expression in the adventitia (open bars) was low in all age groups. Expression of α-smooth muscle actin was significantly greater in the medial layer compared to the adventitial layer at all time points. Data are expressed as mean ± SEM. Data were analyzed by ANOVA with a Tukey's post-hoc test. *$p<0.05$ vs. 15wks group, #$p<0.05$ vs. corresponding adventitia; N=7 per group.

Similar to the expression of α-SMA in the aorta, expression in the mesenteric arteries was localized primarily to the medial layer, with comparatively much less

expression displayed in the adventitia (Figure 46).

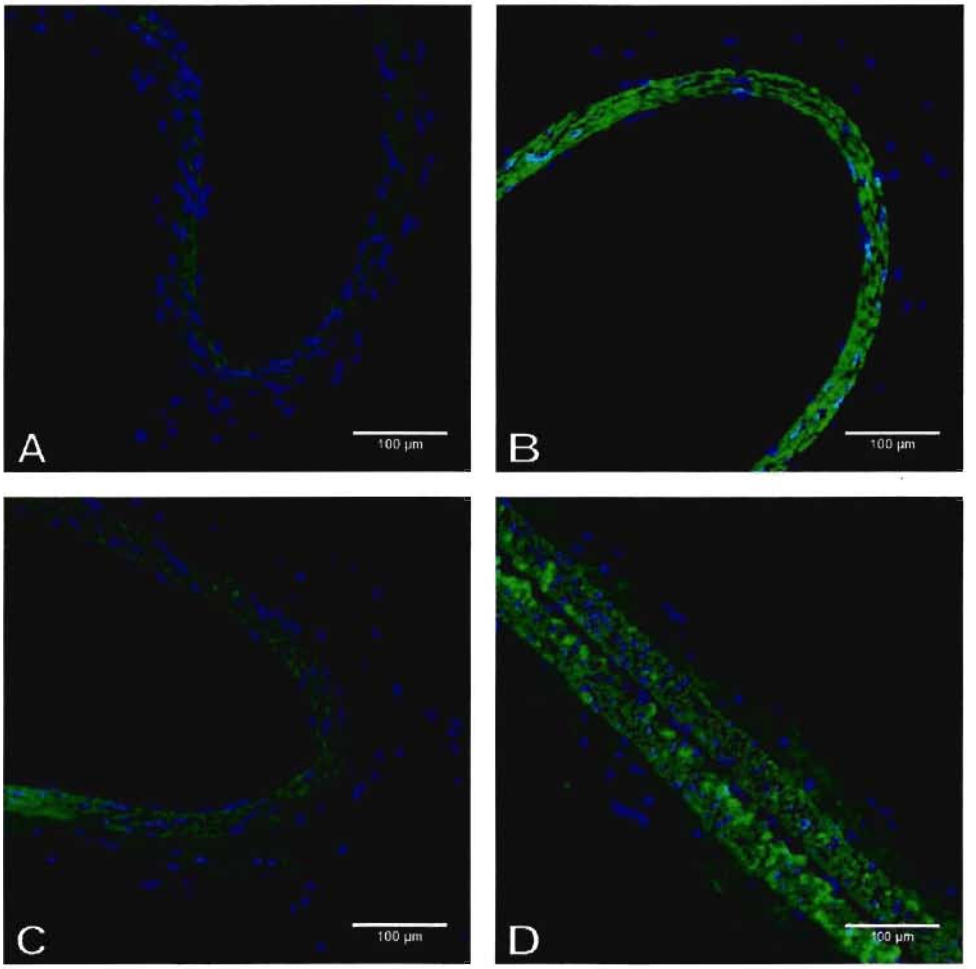

Figure 46. α-Smooth muscle actin protein localization in the mesenteric artery wall. Rat mesenteric arteries aged 15 weeks (A), 30 weeks (B), 50 weeks (C) and 80 weeks (D) expressed α-smooth muscle actin primarily in the media, but only sporadically in the adventitia. Mesenteric arteries were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for α-smooth muscle actin and detected with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

5: Discussion

The findings of this study have positioned the vascular adventitia as a major player in the mediation of age-related vascular diseases. By far the most intriguing finding of this study is the extremely high expression of ECE-1 in the adventitia, raising the possibility that the adventitia is capable of expressing and releasing more ET-1 than any other vascular compartment, including the endothelium. Furthermore, the majority of ET-1 immunoreactivity was found in the medial layer, suggesting that ET-1 produced by the adventitia is capable of migrating to the media, where it may presumably signal vasoconstriction, or growth responses. Additionally the ET receptor heterogeneity found in the vessel wall was quite unexpected while also tremendously remarkable. The majority of ET_A receptor immunoreactivity was found in the media, as expected, but a high amount of ET_B receptor immunoreactivity was found in the adventitia, suggesting a role for the adventitia in vascular ET-1 regulation. Taken together, these results have revealed the adventitia to be a potentially vital tissue in the mediation of vascular disease.

Cell marker analysis of the tissue sections has revealed that the adventitial layer is not comprised of significant amounts of stem cells, endothelial cells, smooth muscle cells, myofibroblasts, or macrophages. Thus, it is likely that the adventitial layer of these tissue sections is comprised primarily of fibroblast cells. Most notably this study has demonstrated that the rat vascular adventitia is capable of growing at a rate that is equal to or greater than the growth of the vascular wall, as well as the medial smooth muscle layer over an 80 week time span. Thus, the proteins and factors expressed by the adventitia may have an equal impact at young ages as in old ages. In addition this study has clearly demonstrated the robust expression of ET system proteins in the vascular

adventitia, and implicated the adventitia in the maintenance vascular tone. Furthermore, this study has shown a steady increase in ECM deposition over the course of the 80 week time span, emphasizing the known contribution of the adventitia to vascular structure, but also highlighting the adventitia's capability to affect vascular stiffness.

5.1 Vascular Structural and Morphological Changes during Aging

5.1.1 Aorta

Analysis of aortic sections revealed overt structural changes in the vessel wall with increasing age. As shown in Table 1, the aortic wall significantly increased in thickness and CSA with increasing age both in terms of the adventitial and medial layers. This thickening eventually led to an increase in wall to lumen ratio at 80 weeks (Table 1), despite the fact that lumen diameter actually increased significantly over that time. This is consistent with previous literature regarding the large central elastic conduit arteries, which exhibit increased wall thickness, CSA and stiffness as well as increased lumen diameter with increasing age (Hannan, et al., 2010; Hashimoto & Ito, 2009). Although measurement of arterial stiffness is not possible in these tissue sections, it would be easy to speculate that such a dramatic increase in wall thickness would accompany a corresponding increase in stiffness of the aorta. Similar studies of arterial aging have shown an increase in large artery stiffness with similar thickening of the arterial wall (Blough, Rice, Desai, Wehner, & Wright, 2007; Santelices, Rutman, Prantil-Baun, Vorp, & Ahearn, 2008).

Of note in this study is that the contribution of the adventitia to aortic wall

thickening with aging has been shown, without focussing solely on the contribution of the intima-media. These data show clearly that the adventitial layer of the aortic wall increases in thickness at a rate that matches the growth of the medial layer (Table 1). Many sources have suggested that the increase in large artery stiffness with increasing age is directly related to a thickening of the intima-media, in addition to structural changes in the organization of the medial layer (Hashimoto & Ito, 2009; Nichols, et al., 2008; O'Rourke & Hashimoto, 2007). With similar morphological changes occurring in the adventitia with increasing age, it may be argued that the aortic adventitia contributes to age-related central artery stiffness in equal magnitude as that of the medial layer. (Hannan, et al., 2010)

5.1.2 Mesenteric Arteries

Unlike the aorta, the morphological changes in the mesenteric arteries with increasing age did not occur in a consistent fashion. The overall increase in wall thickness and CSA of the mesenteric arteries from 15 to 80 weeks of age did not follow the same consistent pattern that was seen in the aorta. An overall increase in wall thickness and lumen diameter in the mesenteric arteries with increasing age has been demonstrated before (Laurant, et al., 2004; Moreau, et al., 1998), however it has been noted that the small muscular mesenteric arteries experience less strain in aging as do large elastic conduit arteries and thus manifest less overt morphological changes over time (Hashimoto & Ito, 2009). This discrepancy is likely due to the difference in elastic properties between these two types of vessels, with the small muscular arteries possessing more distensibility than the large elastic arteries, and are thus subject to less cyclic strain

over a given period of time (Hashimoto & Ito, 2009).

Another possible explanation for the discrepancy in mesenteric artery morphometry with aging may relate to the heterogeneity of the animals used. Although it is generally assumed that a rat of a particular strain will be the same no matter when it was obtained, this simply is not the case. Since each of the separate age cohorts were obtained and raised at separate times, there may exist some differences between individual groups of animals. It has been shown previously that male Sprague-Dawley rats exhibit large variation in avoidance behaviour (Cure & Rolinat, 1992), spatial memory (Ghirardi, Giuliani, Caprioli, Ramacci, & Angelucci, 1992), and anxiety response (Prasad, Henry, & Prasad, 1996). Although no research has examined possible cardiovascular aging heterogeneity in these rats, it remains a definite possibility, and may explain the discrepancy of the changes observed in the mesenteric artery wall in this study.

Also unlike the aorta, the mesenteric arteries exhibited a much different relationship in adventitia to media thickness ratio with increasing age. Whereas the aortic adventitia to media thickness ratio remained constant from 15 to 80 weeks of age, the mesenteric adventitia to media thickness ratio increased significantly from 15 to 30 weeks, and then fell below the control level, reaching a significant difference at 80 weeks of age (Table 2). This perhaps illustrates a very basic difference between the central elastic conduit arteries and the peripheral muscular arteries. The central elastic conduit arteries are subject to much greater cyclic strain over a given time period, and thus require a greater reserve of extracellular matrix in the adventitia to handle the stretch stimulus effectively. Thus, the aortic adventitia grows with the rest of the vessel wall and

the lumen diameter to accommodate this enhanced strain over time. The muscular mesenteric arteries experience far less cyclic strain, and thus have less need for a robust supply of ECM from the adventitia. Despite a slower rate of growth for the mesenteric adventitia when compared to the medial layer, the adventitia was significantly thicker at all time points. Coupled with previous findings showing an increase in the number of mesenteric artery adventitial cells with aging (Briones, et al., 2007), and hypertension (Kantachuvesiri, et al., 2001), this would suggest another important role for the mesenteric artery adventitia.

5.1.3 Pudendal Arteries

The morphological changes seen in the pudendal arteries with increasing age are similar to those seen in the aorta and mesenteric arteries, exhibiting an overall increase in wall thickness and CSA over time. The change in wall to lumen ratio from 15 to 80 weeks was most significant in the pudendal arteries, yielding aged vessels which have grown at a rate far exceeding the growth of the lumen diameter. This can likely be explained by the concomitant rise in mean arterial pressure in the large elastic and small muscular arteries preceding the small resistance vessels such as the pudendals. In order to maintain steady blood flow to organs and tissues, the resistance vessels must dampen the pressure entering these vessels and acutely control it through vasoconstriction (James, et al., 1995; O'Rourke & Safar, 2005). This would explain the massive increase in medial wall thickness and CSA with increasing age, as the acute control of vasoconstriction is performed by the medial smooth muscle.

Despite the tremendous increase in medial wall thickness with increasing age, the

pudendal artery adventitia exhibits no significant change in thickness over time (Table 3). However, adventitia CSA increased slightly over time, showing that the pudendal adventitia does not remain completely dormant. This is consistent with previous findings which showed that the small resistance vessels are capable of maintaining a high degree of arterial compliance in the face of elevated distending pressure through smooth muscle hypertrophy rather than collagen deposition (Hayoz, et al., 1992; Laurent, 1995; Laurent, et al., 1994; Mourad, Girerd, Boutouyrie, Safar, & Laurent, 1998). Thus it is likely that the morphological changes seen in the pudendal artery wall with increasing age reflect this pathway with a tremendous increase in medial layer thickness, but no such change in adventitial layer thickness.

5.1.4 The Arterial Tree

On a whole the data presented in this study illustrate the interconnectedness of the arterial tree along with its distinctly different structural regions including the central elastic conduit arteries, the peripheral muscular arteries, and the small resistance vessels. The pressure load increase experienced in aging leads to a decrease in compliance and resistance of the central elastic conduit arteries which is manifested here in terms of an increase in both aortic wall thickness and lumen diameter. This in turn results in greater flow and pressure exerted on the peripheral muscular arteries, which adapt by increasing wall thickness, a by-product of which is decreased compliance (Mitchell, 2008). This results in higher distending pressure exerted on the small resistance vessels, which compensate through medial smooth muscle hypertrophy in order to provide enhanced temporal control of local blood flow while maintaining normal distensibility (Mitchell,

2008). However, the increased resistance to blood flow produced here results in a larger reflected wave of pressure in the more proximal vessels. Combine this with the enhanced stiffening of these large vessels, and the reflected wave travels fast enough to arrive in close proximity to the forward wave, resulting in a huge increase in systolic and pulse pressures. These in turn drive the 'vicious cycle' forward, further exacerbating the structural changes displayed here (Laurent, Briet, & Boutouyrie, 2009).

The role of the vascular adventitia in this process can be seen in a gradient of influence from a high degree in the elastic aorta, to a lesser extent in the muscular mesenteric arteries, to a near nil effect in the resistant pudendal arteries. This is evidenced by the change in adventitial wall thickness and adventitia to media thickness ratio with increasing age in each of these vessel types. In the aorta, the adventitia grows at a consistent rate over time, matching the rate of growth of the medial layer, likely reflecting the need of the aorta for reinforcing extracellular matrix structural support produced by the adventitia. In the mesenteric arteries, this same relationship is seen early on, from 15 to 30 weeks of age, but it reverses thereafter, with the medial layer outpacing the growth rate of the adventitia. It is possible that these vessels are capable of adapting to the increased pressure load first by reinforcing extracellular matrix components to maintain integrity of the vessel wall in the face of substantial cyclic stretch, but then change this adaptation to medial smooth muscle hypertrophy in order to maintain distensibility and thus flow to adjacent capillary beds and vital organs. In the pudendal arteries, this relationship is shifted entirely towards VSMC hypertrophy, resulting in much faster growth of the medial layer as compared to the adventitial layer over time. However, it should be noted that with the absence of the 30 and 50 week time points in

these vessels, it would be impossible to ascertain if this relationship is consistent from 15 to 80 weeks of age, or is in fact very similar to the peaked relationship seen in the mesenteric arteries.

5.2 The Role of Cell Hypertrophy, Proliferation and Apoptosis During Vascular Aging

This study has shown that VSMCs and ECs are not the only vascular cell types that undergo hypertrophy with aging, and in fact the adventitial cells (most likely fibroblasts) also grow in overall size with increasing age. Additionally, aortic adventitial cells appear to proliferate at a higher rate than any other vascular cell, and do not undergo significant apoptosis, yielding a more substantial adventitial layer later in life.

5.2.1 Cell Hypertrophy

5.2.1.1 Aorta

Aortic medial and adventitial cells increased in overall size with increasing age. In both cases, the increase in size was biphasic, first increasing significantly from 15 to 30 weeks of age, before dropping slightly at 50 weeks, and then rising again at 80 weeks (Figure 5). The increase in aortic cell size follows a similar increase in aortic wall thickness both in the adventitial and medial layers. It has been previously established that VSMCs undergo hypertrophy in aging (Dinenno, et al., 2000; Izzo & Mitchell, 2007), and hypertension (Laurent, 1995; Mourad, et al., 1998). Additionally it has been shown that the adventitia displays an overall increase in the number of cells residing in that layer

with increasing age (Briones, et al., 2007). However, the novelty of this study is in the demonstration of hypertrophy occurring in the adventitia. The logical cause of this phenomenon is likely the increase in cyclic strain experienced in the aorta, leading to damage of the elastic lamellar units, and an increase in ECM deposition by the adventitia to compensate. A resultant increase in adventitial hypertrophy is merely a symptom of the disease.

5.2.1.2 Mesenteric Arteries

The medial and adventitial cells of the mesenteric arteries also exhibit some hypertrophy with increasing age, but with a different pattern from the aorta. As in the aorta, the cells of the mesenteric artery adventitia also exhibit a biphasic increase in overall size with increasing age. However, the mesenteric adventitial cells reach their maximal size at 30 weeks whereas the aortic adventitial cells do not reach their maximal size until 80 weeks of age. The medial mesenteric artery cells, on the other hand, remain at an equal size from 15 to 50 weeks of age before growing significantly at 80 weeks. Again, the changes in overall cell size closely follow the changes in wall thickness in the mesenteric arteries. The mesenteric arteries appear to exhibit a biphasic response to the increased pressure load experienced in aging. This would appear to be in agreement with previous findings of increased contractile sensitivity of the mesenteric arteries in very old rats (Blough, et al., 2007; Moreau, et al., 1998). This is also in agreement with a previous finding showing a recovery in the elastic modulus of the aorta between old and very old rats (Laurant, et al., 2004), presumably to enhance distensibility to better accommodate the increased pressure load seen in aging. Thus, the mesenteric arteries appear to adapt to

increased blood pressure first by reinforcing ECM by the adventitia and secondly by enhancing the medial smooth muscle layer to maintain distensibility.

5.2.1.3 Pudendal Arteries

The pudendal arteries display a small non-significant increase in overall cell size in the adventitial layer, but a greater significant increase in overall cell size in the medial layer (Figure 7). In regards to the adventitial layer, this result is consistent with the observed increase in thickness with increasing age. The pudendal arteries are required to remain distensible in the face of rising arterial pressure, and thus do not display a large increase in adventitial size, which would increase vascular stiffness through the deposition of ECM. The change in medial cell size is also consistent with the increase in thickness of the medial wall; however, with nearly a 4-fold increase in medial wall thickness with increasing age (Table 3), one would expect a larger increase in cell size than that observed (Figure 7). Thus it would appear that the pudendal artery medial wall grows through cell proliferation in aging, rather than by extensive hypertrophy. Although studies regarding the pudendal arteries in aging are sparse, a recent example is consistent with the findings reported here (Hannan, et al., 2010). The pudendal artery is subject to large changes in arterial pressure, especially during copulation, thus the huge increase in medial wall hypertrophy may enable the pudendal artery wall to distend under this high pressure to maintain adequate flow.

5.2.1.4 The Arterial Tree

Overall, the morphological changes observed in each vessel type during aging

relate both to their individual purposes in the vasculature as well as their interconnectedness. The aorta experiences much greater hypertrophy of the adventitial layer which is sustained through the aging process due to the very high pressures exerted on it, and the subsequent damage inflicted on the medial smooth muscle from cyclic strain. The mesenteric arteries experience a somewhat biphasic response to aging, initially displaying a lot of hypertrophy in the adventitial layer, which then declines and allows the lagging medial layer hypertrophy to overtake it. The initial increase in adventitia is likely caused by the large increase in mean pressure from the stiffened aorta by much the same mechanism as in the aorta itself. However, as the animal ages, the need for the mesenteric arteries to distend in the face of chronically elevated arterial pressure is apparent and the medial smooth muscle grows to meet this need accordingly. In the pudendal arteries, the need for distensibility is ever apparent, even at relatively younger ages, thus the increase in medial wall thickness and cell size is more dramatic than in the mesenteric arteries, while little change is observed in the adventitial layer. However, it should be noted that the changes in the pudendal arteries may be occurring in a similar manner as in the mesenteric arteries, but without the intermediate time points available in these vessels this aspect is impossible to determine.

5.2.2 Cell Proliferation

The expression of the cell proliferation marker Ki67 displayed a significant rise with increasing age in the aortic adventitial layer (Figures 9 & 10). More interesting is the fact that expression of Ki67 was localized primarily to the adventitial layer, exhibiting very little expression in the medial layer. This data is consistent with the morphological

changes seen in the aortic adventitial layer with increasing age, which exhibits an age-dependent rise in thickness (Table 1) and cell size (Figure 6). This data is again consistent with previous findings which suggest a thickening and stiffening of the aorta in aging as a compensatory mechanism to adapt to rising cyclic strain over time (Astrand, Ryden-Ahlgren, Sandgren, & Lanne, 2005; Blough, et al., 2007; Mitchell, 2008). The rising expression of Ki67 with aging merely reflects another mechanism by which the aortic wall compensates for and adapts to the age-dependent rise in arterial pressure and wall stress.

5.2.3 Cell Apoptosis

Defying all expectations, the aorta sections examined here exhibited almost zero expression of the apoptosis marker cleaved caspase-3. Apoptosis is a very common process in numerous aging cell populations (Asai, et al., 2000; Hoffmann, et al., 2001; Intengan & Schiffrin, 2001; Torella, et al., 2004). Furthermore, previous research has found that aging increases the sensitivity of aortic VSMCs (Schauer, et al., 2010) and ECs (Hoffmann, et al., 2001) to apoptosis or apoptotic stimuli. So it would seem that the data presented here do not represent the consensus knowledge on apoptosis in arterial aging. However, in many studies examining apoptosis, acute injurious stimuli are utilized to induce changes in gene expression, which often lead to apoptosis. In this study, no such stimuli were experienced, and the tissue displayed here exhibit the result of chronic stress over a long period of time. Additionally, the process of apoptosis is ongoing in all tissues, not just in aging, and thus may not be fully described in isolated single time points such as in this study.

Further examination with the caspase-3 antibody showed a similarly low amount of apoptosis in aorta sections isolated from a hypertensive mouse (Figure A-4), a model known to induce apoptosis (Intengan & Schiffrin, 2001). Thus, it is likely that the method used to detect apoptosis is not sufficiently sensitive. Alternatively, these tissues could be undergoing apoptosis that is caspase-3 independent, although previous attempts at detecting apoptosis using the TUNEL assay were unsuccessful as well.

5.2.4 The Interplay of Cell Hypertrophy, Proliferation and Apoptosis in Vascular Aging

Overall, the changes observed in the aging vasculature in regard to cell hypertrophy, proliferation and apoptosis reflect the local arterial pressure changes occurring in aging. As blood pressure increases with time due to arterial wall damage and stiffening, the arterial tree adjusts to accommodate these factors. The arterial walls grow thicker, while the cells proliferate and grow in size to better handle the chronic wall stress exerted by high blood pressure. The changes seen in these vessels may seem pathogenic; however they are simply a necessary evil needed to maintain arterial blood flow and perfusion of tissues.

5.3 The Role of Endothelin System Expression in Vascular Aging

This study has shown for the first time an active and strong ET system in the vascular adventitia. Not only do ET system proteins localize to the adventitia, but they are often expressed to a greater degree here than in the intimal or medial layers. Given the tremendous influence of ET-1 in the vasculature on cell growth, proliferation, and

vascular tone, the implications of these data are potentially quite profound.

5.3.1.1 ET-1

ET-1 expression in the aorta, mesenteric arteries, and pudendal arteries was spread throughout the vessel wall, with somewhat greater localization to the medial layer (Figures 12, 20, & 28, respectively). In the aorta, with increasing age, ET-1 expression remained constant in the medial layer, but fluctuated in the adventitial layer (Figure 13). A similar pattern of expression was found in the mesenteric arteries with increasing age (Figure 21). These data present interesting evidence since the antibody used to detect ET-1 expression detects the BigET-1 isoform of ET-1 specifically. The lower amount of detectable BigET-1 in the adventitia likely reflects the enhanced ability of this tissue to cleave BigET-1 into the mature ET-1 peptide, given the high expression of ECE-1 protein in the adventitia. Thus although it would appear expression of ET-1 in the adventitia is lower than in the media, taking all factors into account would suggest that the adventitia is a more efficient cellular factory of mature ET-1 than the media. However, it is interesting to note that expression of ET-1 in the medial layer does not change with increasing age in the aorta and mesenteric arteries, suggesting ET-1 is necessary for maintaining vascular tone and cell growth in these vessels, which have been demonstrated previously (Haynes, et al., 1996; Iemitsu, et al., 2006; Ivey, Osman, & Little, 2008; Rodriguez-Vita, et al., 2005). This would also be consistent with the morphological data presented in this study, which show that the aortic and mesenteric artery walls grow at a steady rate with increasing age, rather than reaching a maximum and levelling off. Unfortunately it is impossible to measure vascular contractile and

dilatory responses in these sections, but previous studies have shown an alteration in ET-related vasomotor responses in aging (Donato, Lesniewski, & Delp, 2005; Korzick, et al., 2005).

In the pudendal arteries, ET-1 expression is relatively high in the media and adventitia of the 15 week old sections, before dropping off completely at 80 weeks (Figure 29). This is in agreement with the ET_A receptor data from these vessels (Figure 33), and morphological data (Table 2), which show a tremendous increase in the medial smooth muscle layer and concomitant drop in ET_A receptor expression. The protein expression changes are likely a functional adaptation to the morphological changes since a high degree of ET-1 and ET_A receptor expression would result in a high degree of vasoconstriction given the thickened medial smooth muscle layer. Alternatively, a high production and release of mature ET-1 would drive down expression of the ET_A receptor through a negative feedback mechanism.

Previous data from our lab has shown that ET-1 is involved in type I procollagen synthesis in cultured adventitial fibroblasts (An, et al., 2006). Additionally, it has been shown that ET-1 can stimulate contractile responses in a tissue-engineered blood vessel model comprised entirely of adventitial cells (Laflamme, et al., 2006). Thus the presence of the ET-1 peptide in the adventitial layer may serve to activate these pathways, combining to stiffen and strengthen the aortic wall in the face of rising arterial pressures. The fact that detectable ET-1 declines with aging in the adventitia may be an artefact of the tissue isolation and preservation procedure, or may reflect the clearance mechanism exhibited by the high expression of the ET_B receptor in the adventitia of the aorta, mesenteric arteries, and pudendal arteries (Figures 18, 26, & 34, respectively).

5.3.1.2 ECE-1

The most striking result of this study lies in the localization of ECE-1 protein to the vascular adventitia of the aorta, mesenteric arteries, and pudendal arteries, as shown in Figures 14, 22, and 30, respectively. It has long been established that ET-1 is produced by vascular endothelial cells and that ECE-1 is highly expressed in these cells (Hickey, et al., 1985; Yanagisawa, et al., 1988). This is consistent with the present data, which shows a thin band of ECE-1 localization in the endothelium. However, expression of ECE-1 in the endothelium is inconsistent since many sections appear to be lacking endothelial cells, which may have been removed during the isolation procedure, or by high arterial pressures. Nevertheless, expression of ECE-1 is still greater in the adventitia, even when intact endothelium is present.

Localization of ECE-1 to the adventitia is consistent with our previous data showing expression and release of ET-1 from cultured adventitial fibroblasts (An, et al., 2006). However, our data would suggest that the amount of ET-1 that adventitial fibroblasts express and release is similar to previously published figures for VSMCs (A. W. A. Hahn, et al., 1990), but far less than figures published for ECs (Ozaki, et al., 1995). This would appear to be in contrast to the data presented here, which would suggest that the adventitia, above all other vascular wall components, possesses the greatest capacity for expressing and releasing ET-1. Of course this discrepancy could be explained by differences between the previously published *in vitro* data and the *in vivo* data presented here. It is possible that in a monoculture of adventitial fibroblasts, with the absence of a paracrine signal arising from the endothelium or medial smooth muscle, the adventitial cells simply have no need for synthesizing and releasing ET-1. On the other hand,

expression of ECE-1 does not necessarily correlate with ET-1 release, although expression of such a high level of ECE-1 would be quite energetically wasteful if it did not serve such a purpose. It should be noted that with such a high level of expression of ECE-1, the adventitia may possess the capability of controlling ET-1 content of the vascular wall, and have the ability to override ET-1 secretion by any other vascular wall component. Whereas the consensus knowledge would contend that the vascular endothelium is the main source of ET-1 in the vasculature, these data would suggest that the adventitia can overcome the endothelium in this regard.

5.3.1.3 ET_A

Although expressed in the adventitia, the expression of the ET_A receptor was localized primarily to the medial layer of the vessels examined (Figures 16, 24, & 32, respectively). In the aorta, expression of the ET_A receptor was consistently low in the adventitia from 15 to 80 weeks of age. On the other hand, expression of the ET_A receptor in the medial layer rose substantially, at 80 weeks (Figure 17). Meanwhile in the mesenteric arteries, expression of the ET_A receptor fluctuated with increasing age in both the medial and adventitial layers (Figure 25). And finally, expression of the ET_A receptor in the pudendal arteries displayed a sharp decline from 15 to 80 weeks in both the medial and adventitial layers (Figure 33) Given the substantial growth of the adventitia with increasing age, it is surprising not to find more ET_A receptor protein localized to the adventitia since it mediates the main mitogenic responses of ET-1 (Rodriguez-Vita, et al., 2005; Shi-Wen, et al., 2001). It is entirely possible that the growth and proliferatory responses observed in the adventitia with aging are mediated by mitogenic signalling

molecules other than ET-1, or these responses are stimulated simply by the wall stress exerted by elevated arterial pressure.

On the other hand, ET_A receptor expression in the medial layer of the aorta and mesenteric arteries is consistent with previous findings which suggest an increase in ET_A-dependent vasoconstriction in aged vessels (Thijssen, Rongen, van, Smits, & Hopman, 2007; Van Guilder, et al., 2007). This would also contribute to an increase in aortic wall stiffness if the baseline ET-1 related vascular tone is in fact elevated. Additionally, the enhanced expression of the ET_A receptor with increasing age may relate to connective tissue formation in the medial layer (Rodriguez-Vita, et al., 2005). This may occur in response to the damage of medial lamellar units during aging, a process which is quite common in vascular aging (Connat, et al., 2001). Furthermore, it has been found that the ET_A receptor may mediate EC adhesion molecule expression, which is caused by vascular inflammation, a process likely to be involved in vascular aging (Callera, et al., 2004). Thus, with many cellular responses mediated by an ET_A receptor pathway, even a relatively mild change in ET_A receptor expression can have a considerable effect on the vascular wall. Moreover, with such varying responses mediated by this pathway, a similar change in expression in opposing tissues or cell types can have vastly divergent effects. In the pudendal arteries, the decline in ET_A receptor expression is likely a functional adaptation to the massive hypertrophy of the vessel wall to maintain a patent dilated vessel under resting conditions.

5.3.1.4 ET_B

Expression of the ET_B receptor localized partly to the endothelium, but primarily

to the adventitial layer of the aorta, mesenteric arteries, and pudendal arteries (Figures 18, 26 & 34, respectively). It has been previously established that the ET_B receptor mediates vasodilation (Hirata, et al., 1993), ET-1 synthesis down-regulation (Ozaki, et al., 1995), clearance of circulating ET-1 (Fukuroda, et al., 1994), and inhibition of NADPH oxidase (J. K. Dammanahalli & Z. Sun, 2008) in ECs. It is likely that at least some of these functions are present in adventitial cells. Coupled with the simultaneous high expression of ECE-1 in these locations, the logical role for the ET_B receptor is likely in a negative feedback or clearance mechanism.

Interesting to note here is that the ET_B receptor is expressed to a much greater extent than the ET_A receptor in the adventitia. This would appear to be in contrast to previous findings from an adventitial cell-derived tissue engineered blood vessel construct (Laflamme, et al., 2006), and data from our own lab in cultured adventitial fibroblasts (An, et al., 2006), which each show a greater abundance of the ET_A receptor than the ET_B receptor. In each of these cases, however, the system being used represents a monoculture of adventitial fibroblasts, and thus would lack any potential paracrine signals arising from neighbouring cell types. Such potential paracrine signals may be necessary for adventitial fibroblasts to express a significant detectable amount of the ET_B receptor, and thus may explain the discrepancy in findings.

5.3.4 Overview of the ET System in the Arterial Tree

The findings of the present study in regards to ET system expression are very striking. For the first time, the present study has demonstrated the robust expression of ET system proteins in the vascular adventitia. Additionally, these proteins are not limited

to a single vascular bed, as each of the thoracic aorta, mesenteric artery and pudendal artery adventitial layers express similar amounts of all 4 ET system proteins. Most striking is the expression of ECE-1 to such a great degree in the adventitia. Conventional wisdom has maintained that the endothelium contains the highest concentration of ECE-1 protein, and is capable of releasing the most ET-1 of any cell type in the vascular wall (Ozaki, et al., 1995). The results of the present study would appear to refute that claim, and would suggest that the adventitia possesses the capability to synthesize and release far more ET-1 than any other vascular cell type. Considering the activity of ET-1 in controlling vascular tone by acting on VSMCs (Haynes, et al., 1996), from an architectural standpoint, this finding would make perfect sense. It is unlikely that the endothelium, comprised of cells that are often subjected to injurious stimuli based on their location, would alone be capable of synthesizing and releasing enough ET-1 to control vascular tone simply because it is adjacent to the medial layer. Thus it only makes sense that the adventitia would possess a similar mechanism, also residing adjacent to the medial layer.

5.4 The Roles of ROS Content, ECM Expression and Myofibroblast Induction in Vascular Aging

The processes of ROS production, ECM expression, and myofibroblast induction are generally thought to negative consequences of cellular damage repair. The results of the present study have presented conflicting data which both support and refute this idea, revealing that these processes may not be clear in their purposes.

5.4.1 ROS Content

Although one would expect oxidative stress to increase dramatically with age, the data presented here do not support this hypothesis. Instead, 3-nitrotyrosine, a marker of cellular oxidative stress displayed its greatest expression between 15 and 50 weeks, and declined at 80 weeks, although not significantly (Figure 37). Under identical experimental conditions, a significant amount of 3-nitoryrosine could be detected in aorta sections isolated from an angiotensin II-infused hypertensive mouse (Figure A-5), a model known to induce significant oxidative stress (Wang, et al., 2001). Thus, it is not likely that the probe used to detect ROS in this study was not sufficiently sensitive. It has been commonplace to assume oxidative stress to increase with aging, thus resulting in an accumulation of cellular damage and the common aged phenotype. However, several lines of evidence would seem to refute this claim. First, clinical trials with vitamin E supplementation, a powerful antioxidant, have resulted in very little positive effect in disease prevention (Sesso, et al., 2008). Second, the paradoxical example of the *mclk+/-* mouse, which exhibits much higher levels of oxidative stress, but lives longer than its wild-type contemporaries (Lapointe & Hekimi, 2008). And finally, results from the present study show a slight decline in overall in collagen type I protein expression after age 15 weeks (Figure 42), which our lab has shown to be a ROS dependent pathway in adventitial fibroblasts (An, et al., 2007). Thus, the present study may represent part of the growing body of evidence refuting a large role for oxidative stress in the aging process.

Interestingly, the localization of 3-nitrotyrosine in the adventitial layer did not differ significantly from the medial layer at any time point (Figure 37). This is in contrast with previous findings showing the adventitia to be a rich source of ROS in the vascular

wall (Pagano, et al., 1997; Wang, et al., 1999; Wang, et al., 1998). Numerous other studies have shown detrimental effects of adventitial-derived ROS in mediating processes such as neointimal formation (Dourron, et al., 2005), hyperplasia (Weaver, et al., 2006), and VSMC hypertrophy (Liu, et al., 2004). However, each of those studies employed acute injurious stimuli to stimulate adventitial ROS production. In the model employed here, the vascular adventitia is not exposed to such a similar acute and intense injury, but rather is exposed to the mild chronic injury of prolonged cyclic strain. It is possible that this weaker stimulus is not sufficient to evoke a large response in terms of ROS production. Similarly, the mechanisms for ROS production, such as NADPH oxidase, often comprise short-acting, inducible enzymes which produce short, transient bursts of ROS. With an isolated view of the vessel wall at individual time points, it is exceedingly unlikely to capture one of these transient processes in action.

5.4.2 Extracellular Matrix Expression

It has long been established that the arterial walls stiffen during aging in a process called arteriosclerosis. One of the common mechanisms for this age-related stiffening is the enhanced expression and deposition of ECM proteins. A common consequence of ET-1 signalling is the production of ECM proteins, such as collagen. Given the immense expression of ECE-1 in the vascular adventitia, the accumulated effects of ET-1 signalling are likely to be seen in the adventitia, displaying enhanced ECM expression during aging. Here, in the present study, the expression of the ECM proteins fibronectin and collagen type I have been explored to address the potential change in ECM protein expression and deposition with increasing age.

5.4.2.1 Fibronectin

Fibronectin expression in the aortic adventitia rose steadily and significantly from 15 to 50 weeks of age before declining at 80 weeks (Figure 39). Meanwhile, expression of fibronectin in the medial layer did not change significantly from 15 to 80 weeks of age (Figure 39). This discrepancy in fibronectin expression between the adventitial and medial layers was fully expected, since fibroblasts produce immense amounts of ECM proteins, compared to other common vascular cell types (Gabbiani, 2003). It has also been shown that fibronectin expression provides a conduit for fibroblast migration in wound repair processes (Briggs, 2005; Greiling & Clark, 1997). Additionally, ET-1 signalling has been shown to stimulate fibronectin expression in cultured skin fibroblasts (Shi-Wen, et al., 2001). Combined with the overall decline in ET-1 expression with increasing age, the probable reason for enhanced fibronectin expression during the aging process would be for injury repair and fibroblast migration.

It is possible that the vessel is sufficiently stiffened and reinforced after 50 weeks to the point that excessive fibronectin expression is no longer necessary. Alternatively the mechanism by which fibronectin expression is activated may be damaged after 50 weeks, thus reducing its overall expression. Further experiments would be warranted to elucidate the specific cause for this change in expression. A similar expression pattern of fibronectin was seen in the mesenteric arteries (Figure 40), suggesting the role of fibronectin in aging is not limited to the aorta.

5.4.2.2 Collagen type I

The expression of collagen type I in the adventitia remained relatively unchanged

from 15 to 80 weeks of age in the aorta (Figures 41 & 42). The mesenteric arteries displayed a similar preferential localization of collagen type I to the adventitial layer, but appeared to show an increased expression at 80 weeks (Figure 43). Numerous studies have shown that collagen expression is stimulated in vascular cells by Ang II signalling (An, et al., 2006; Boffa, et al., 1999; Che, et al., 2008; Ford, Li, & Pickering, 1999). Additionally, ET-1 signalling may also have effects in the stimulation of collagen synthesis (An, et al., 2006; Horstmeyer, Licht, Scherr, Eckes, & Krieg, 2005; Shi-Wen, et al., 2001). The enhanced expression of collagen in the vasculature is a well established phenomenon leading to enhanced age-related vascular stiffness (Izzo & Mitchell, 2007; Mitchell, 2008). However, combined with the morphological data presented earlier, the overall collagen content may actually remain sufficiently high, despite average expression falling with increasing age.

5.4.3 Myofibroblast Induction

The adventitial layers displayed almost zero expression of α-SMA at all ages, suggesting a non-existent level of myofibroblast content (Figure 45). Thus it would appear that myofibroblast induction is not a significant factor in vascular aging because it is commonly known that fibroblasts undergo phenotypic differentiation to myofibroblasts and express α-SMA under high stress conditions such as wound healing (Gabbiani, 2003). It is likely that the process of aging alone does not present a particularly noxious stimulus for the induction of such a phenotypic switch and the adventitial myofibroblast content is unchanged.

5.4.4 The Interplay of ROS Content, ECM Expression, and Myofibroblast Induction in Vascular Aging

Overall one would expect ROS content, ECM expression and myofibroblast induction to be enhanced during aging, resulting in a damaged, stiffened, and reactive aged vascular wall. However, as previous literature has illustrated, the requirement for significant induction of each of these processes include particularly noxious stimuli. It is likely that the process of vascular aging represents a relatively mild chronic injurious stimulus, which is not sufficiently noxious as to induce these energetically expensive processes. The consistent changes in ROS content, ECM expression, and myofibroblast induction further emphasize this point.

5.5 Summary of the Studies

This study has aimed to characterize the vasculature during the process of aging, with a conscious focus on the vascular adventitia. The present study has presented data regarding the age-dependent change in vascular structure, function, morphology, and protein expression in the thoracic aorta, mesenteric arteries, and pudendal arteries. The data presented has demonstrated that the vascular adventitia is an active participant in the extensive changes that occur in the vasculature with increasing age. It has been shown clearly that the vascular adventitia grows in thickness and in overall cell size with increasing age, at a rate that matches the growth of the rest of the vessel wall. Additionally, proteins expressed in the adventitia can potentially influence the rest of the vessel wall not only by providing enhanced stiffness and structural support, but also through the generation of signalling molecules that influence vascular tone, cell growth,

and proliferation. It is clear from the results of this study that the common functional alterations occurring concomitantly with vascular aging can be attributed, at least in part, to the structural and functional alterations of the vascular adventitia.

5.6 Significance of the Studies

The results of this study have clearly described for the first time the structural and functional alterations that occur in the vascular adventitia with aging. Most notably, this study has provided the first direct evidence of the expression of a robust ET system in the vascular adventitia that is capable of controlling ET-1-related vascular tone. Furthermore, the growth of the adventitial layer with increasing age occurs at a rate that matches the growth of the vascular wall, thus allowing the adventitia to influence the structure and function of the vascular wall throughout the process of aging. It is clear from these results that the influence of the vascular adventitia cannot be ignored when considering vascular processes, both in health and disease.

5.7 Limitations and Future Perspectives

5.7.1 Limitations of the Studies

5.7.1.1 Immunofluorescence Detection of Protein Expression in Formalin-Fixed Paraffin-Embedded Tissue Sections

A commonly held perception of molecular biologists is that formalin fixation of tissues precludes the utilization of immunofluorescence as a protein detection method

since aldehyde fixatives cause cross-linking of cellular proteins to enhance stability, but which also leads to a high degree of autofluorescence (Robertson, Savage, Reis-Filho, & Isacke, 2008). This autofluorescence can mask the fluorescence of tagged antibodies bound to proteins of interest, making it very difficult to determine what the real protein expression level of a tissue is. However, numerous studies have published reports of useful, optimized methods for utilizing immunofluorescence in formalin-fixed tissue (Baschong, Suetterlin, & Laeng, 2001; Bataille, et al., 2006; Mason, Micklem, & Jones, 2000; Robertson, et al., 2008; Suetterlin, Baschong, & Laeng, 2004; Viegas, Martins, Seco, & do Carmo, 2007). Furthermore, antigen retrieval techniques can be employed to reverse the cross-linking caused by formalin fixation, thereby eliminating autofluorescence issues. Most commonly this can be achieved through the use of alcoholic or aqueous solvents to reduce the aldehyde cross-link bonds, both of which were employed in this study. Additionally, measures were taken to ensure that the fluorescence observed in the tissues was caused by the fluorescent probe through the use of negative control samples. In this case, normal autofluorescence was reduced to nil, verifying the validity of the method.

5.7.1.2 Protein Expression/ Vessel Function Relationship

Given the chosen method of studying these vessels (formalin-fixed paraffin-embedded tissue sections), it would be impossible to detect changes in vessel dynamics such as constriction and dilation. Therefore, the only functional data we can collect are purely circumstantial and indirect. We can surmise that with an overall increase in the thickness of the vessel wall, and an enhanced expression of key proteins, the vessel

would gain the ability to be far more reactive. However, without direct functional data, these assumptions remain purely speculative. Further study is warranted to determine the direct functional implications of the broad changes observed to occur with increasing age in this study.

5.7.1.3 Isolation of the Vascular Adventitia

A common challenge in studying the vascular adventitia has been the isolation of this tissue from the rest of the vessel wall. Primary cell culture is an attractive means since we and others have shown that adventitial fibroblasts grow well in culture (An, et al., 2006; Che, et al., 2008; Jenkins, et al., 2007). However, with no specific cell marker available to positively identify fibroblast cells in culture, it becomes increasingly difficult to ascertain the true origin of cultured adventitial fibroblasts. Additionally, there are key differences in the activity of adventitial cells *in situ* in the vessel wall and plated in culture, making inferences made about vascular biology from cultured adventitial fibroblasts difficult to make. Furthermore, the close and tight association of the adventitial layer with the rest of the vessel wall makes physical separation exceedingly difficult to perform without damaging important cell structures. Thus, the best method for observing the vascular adventitia available for this study remains formalin-fixed paraffin embedded tissue sections.

5.7.1.4 Potential Confounding Effects of Obesity

The animals used in this study exhibited a steady increase in body weight at each time point examined (Table A-1). Since the animals were given free access to food and

water, it is possible that the older animals may have developed the onset of obesity. However, examining the effects of body weight independently of age is not possible in this study since the age and body weight of the animals are so highly correlated that examining both age and body weight in one statistical model would result in a high degree of collinearity and highly unstable estimates of true effect. Unfortunately this is a limitation of the study design, and in order to examine the independent effects of age and body weight, one would need separate cohorts of obese-prone and obese-resistant animals at each time point. Alternatively, caloric restriction could be employed for sub-groups of animals to achieve a wider variation in body weight among each of the individual age cohorts.

5.7.2 Future Perspectives

5.7.2.1 Caloric Restriction in Aging

Caloric restriction has been shown by numerous studies to prolong lifespan, improve cellular oxidative damage, and result in far less age-related pathology (Choi & Yu, 1998; Csiszar, et al., 2009; Hannan, Heaton, & Adams, 2007; Masoro, 2005). In addition, caloric restriction has been shown to have a positive impact on such important cardiovascular factors such as endothelial function (H. Yang, Shi, Story, Richardson, & Guo, 2004), cerebral blood flow (Lynch, et al., 1999), age-related arteriosclerosis (Chiarpotto, Bergamini, & Poli, 2006), and serum prostaglandin expression (Choi & Yu, 1998). Furthermore, it should be noted that the red wine polyphenol resveratrol has been shown to produce similar effects (Chao, et al., 2005; Pearson, et al., 2008). However, the

effects of a caloric restriction regimen on the structure and function of the vascular adventitia has never been addressed. Given the vast changes observed in the vascular adventitia with increasing age in this study, caloric restriction could potentially serve as a simple, effective treatment against the age-related detriments to the vascular adventitia.

5.7.2.2 Adventitial Endothelin System Activity

The present study has provided the first clear evidence of ET system protein expression in the vascular adventitia. However, due to the limitation in the isolation of the adventitia, the present study has not been able to show whether or not this system is definitely active, or furthermore able to influence the rest of the vessel wall. It is possible to constitutively activate or inactivate the expression of endothelin system proteins in a particular tissue or cell type (Amiri, et al., 2004; Kelland, et al., 2010; Kisanuki, et al., 2010). Altering the expression of ET-1 specifically in the endothelium, or vascular smooth muscle, would allow one to determine the net effect of ET-1 expression in the vascular adventitia. Thus, we may be able to gain knowledge into the role of the adventitia in mediating ET-related vascular diseases.

5.7.2.3 Disease Models Exhibiting Enhanced Endothelin System Expression

Given the present demonstration of intense expression of ET system proteins in the vascular adventitia, one can only wonder as to the impact that the adventitia may have on ET- related vascular diseases. Previous studies have shown an elevation in the expression and release of ET-1 in the DOCA-salt hypertensive rat model (Lariviere, et al., 1995) and the Ang II infused model (Barton, Shaw, d'Uscio, Moreau, & Luscher,

1997). Furthermore, data from our own lab has shown that Ang II stimulation in adventitial fibroblasts induces expression and release of ET-1 (An, et al., 2006). Given these lines of evidence, it would be interesting to learn if ET-1 expression in the vascular adventitia is similarly enhanced *in vivo* in these disease models, which may provide a novel approach to therapy in hypertension.

5.8 Conclusion

The results of the present study have provided a detailed characterization of the vascular adventitia in aging. Combining the structural and functional changes occurring in the aging adventitia, it is clear that the adventitia has the capability to profoundly influence normal vascular function in aging. Additionally, the adventitia is clearly implicated in the initiation and progression of numerous age-related vascular diseases, such as arteriosclerosis and hypertension. Thus it is clear that the vascular adventitia can no longer be ignored when concerning vascular function, both in health and disease.

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Appendix

Table A-1. Body and heart weights.

Measurement	*15 wks*	*30 wks*	*50 wks*	*80 wks*
Body Weight (g)	$347.0 \pm 6.9^{b,c,d}$	$602.7 \pm 9.0^{a,d}$	681.3 ± 24.6^{a}	$765.0 \pm 22.9^{a,b}$
Left Ventricle Weight (g)	$0.70 \pm 0.02^{b,c,d}$	1.04 ± 0.04^{a}	1.06 ± 0.01^{a}	1.13 ± 0.02^{a}
Right Ventricle Weight (g)	$0.19 \pm 0.01^{b,c,d}$	0.29 ± 0.01^{a}	$0.27 \pm 0.01^{a,d}$	$0.30 \pm 0.01^{a,c}$

Mean ± SEM; [a]$p<0.05$ vs. 15 wks; [b]$p<0.05$ vs. 30 wks; [c]$p<0.05$ vs. 50 wks; [d]$p<0.05$ vs. 80 wks

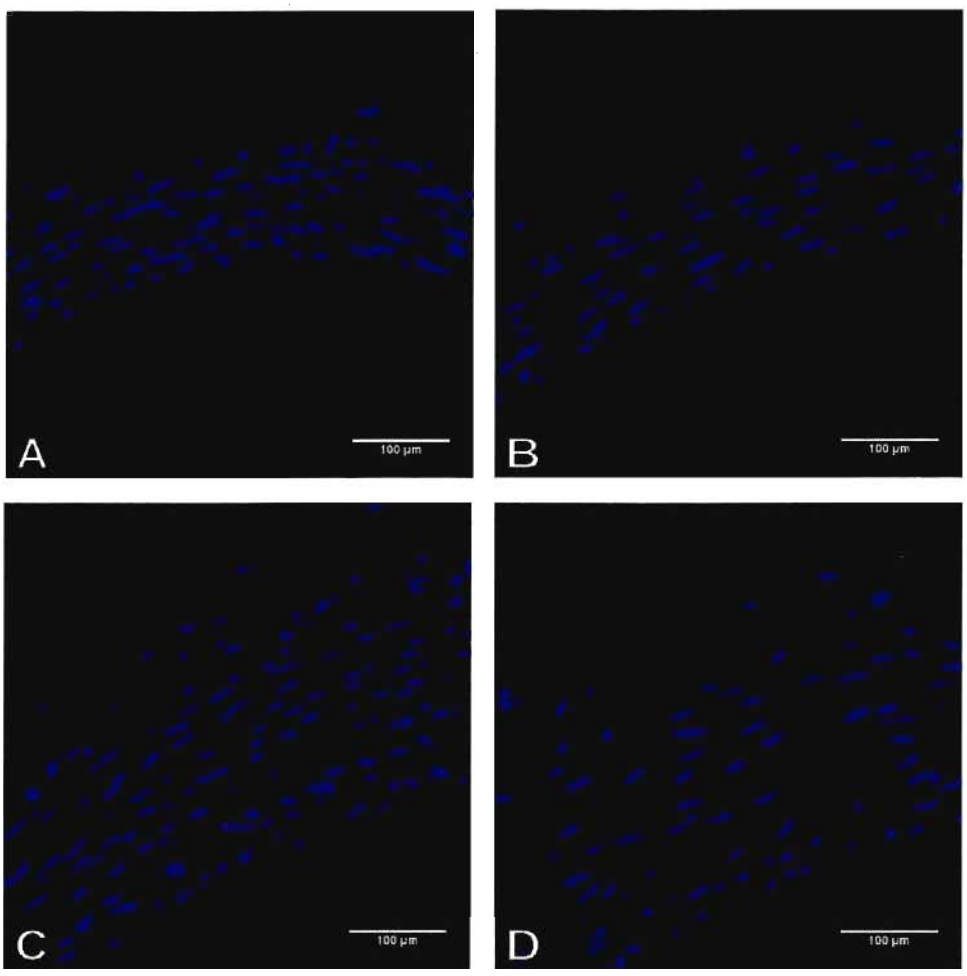

Figure A-1. Immunofluorescence negative control. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) displayed very little non-specific binding of the ET-1 antibody after peptide competition. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then incubated with an anti-ET-1 antibody which had previously been incubated with the ET-1 peptide. Samples were then incubated with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

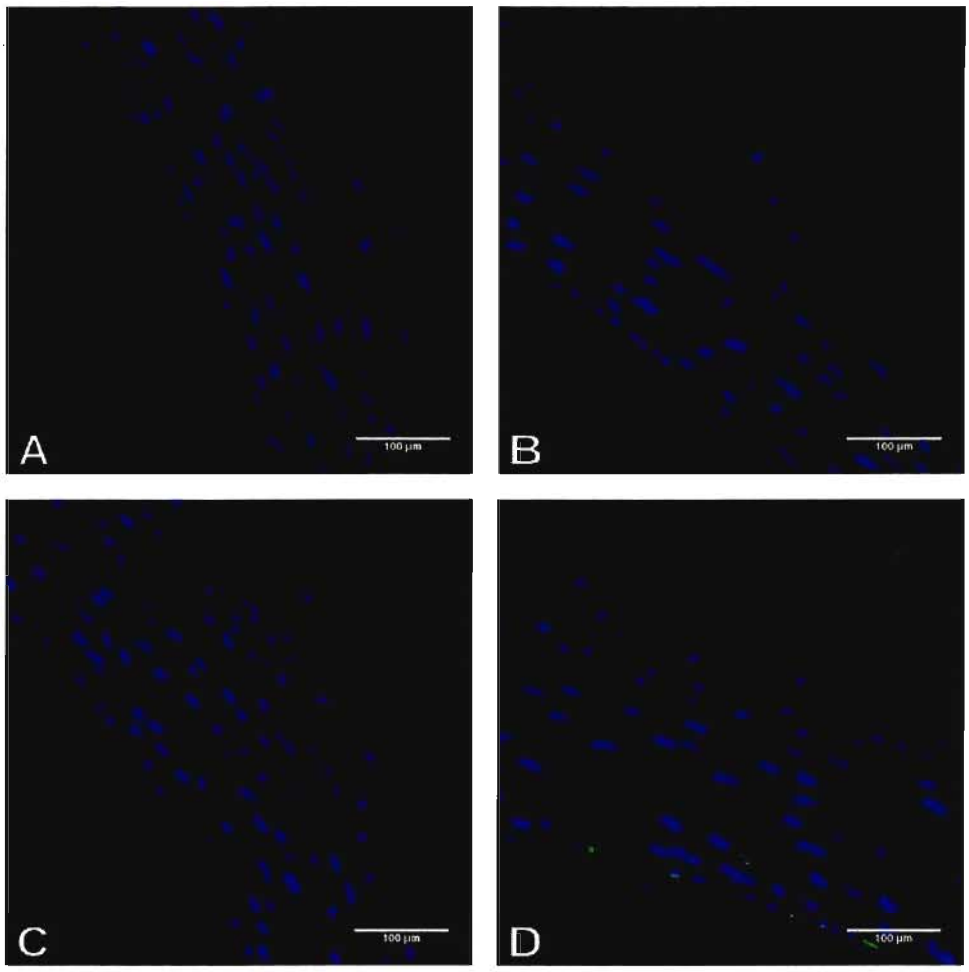

Figure A-2. von Willebrand factor protein localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) displayed some von Willebrand factor (vWF), a specific endothelial cell marker, in the endothelium. vWF was not expressed in either the medial or adventitial layers. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for vWF and detected with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

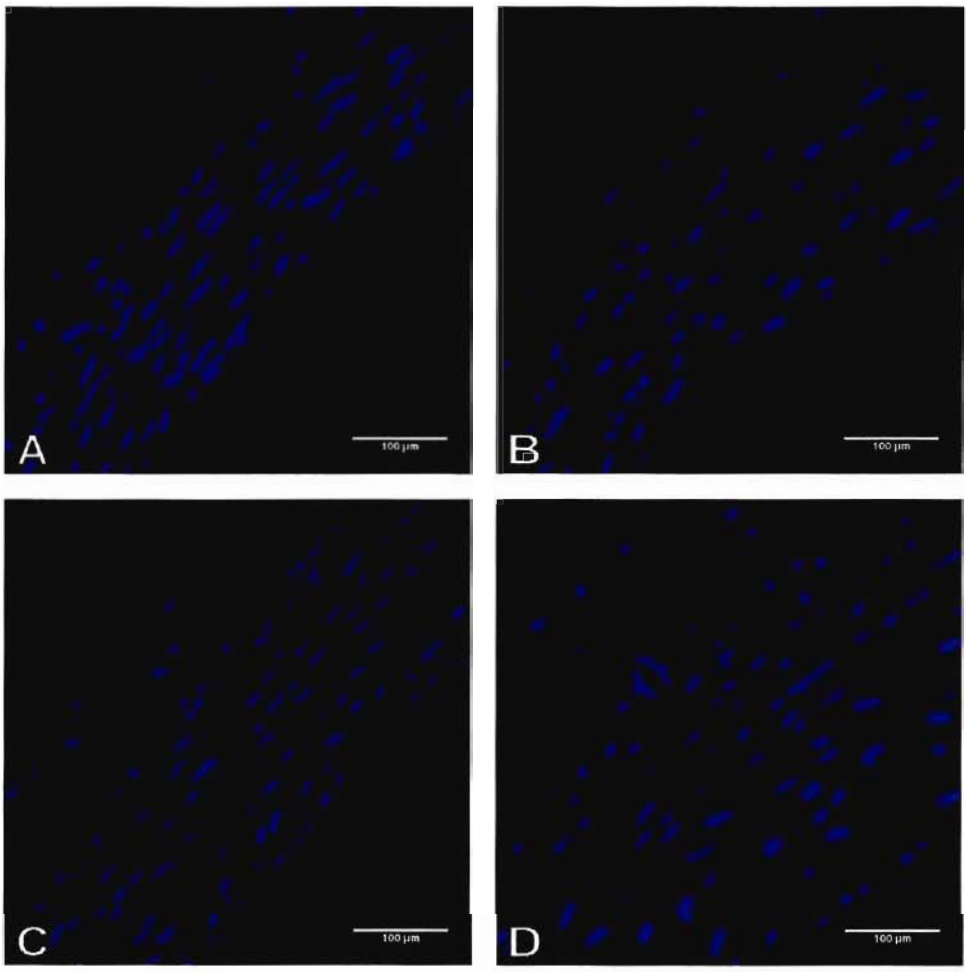

Figure A-3. CD4 protein localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) displayed very little CD4 protein, a specific cell marker for macrophages. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for CD4 and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

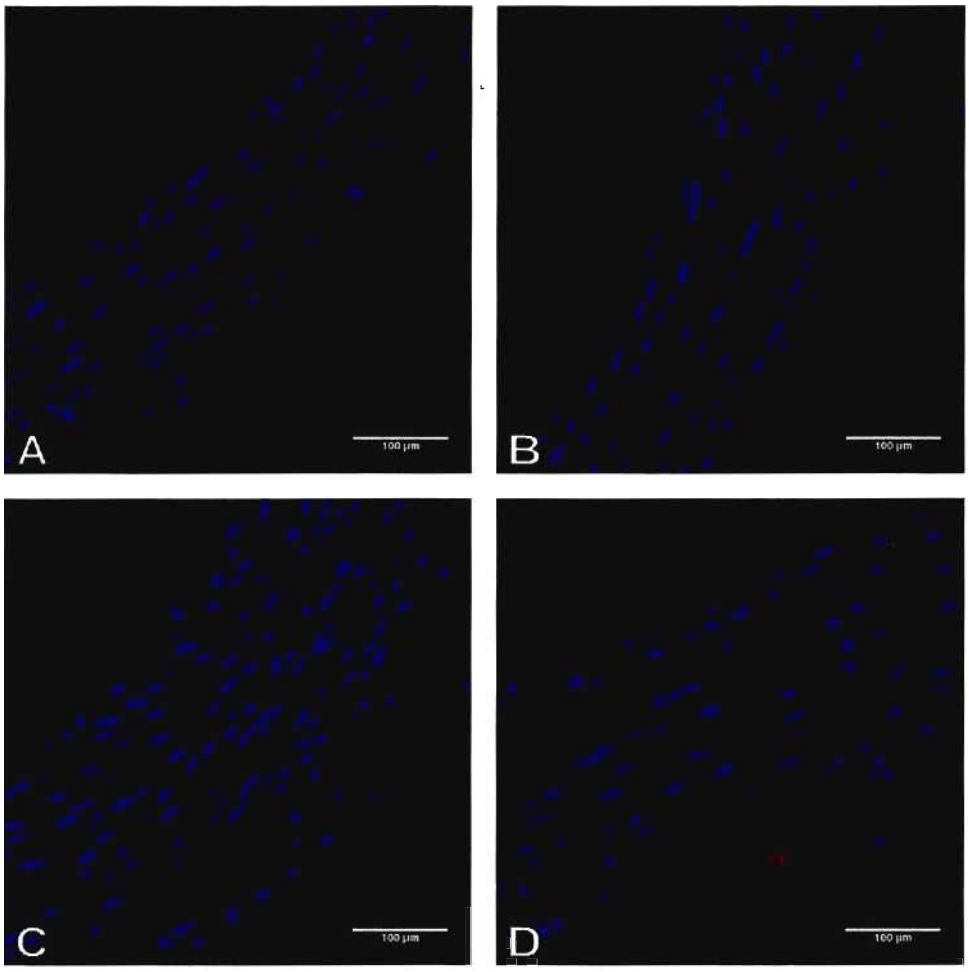

Figure A-4. c-kit protein localization in the aortic wall. Rat aortas aged 15 weeks (A), 30 weeks (B), 50 weeks (C), and 80 weeks (D) displayed very little c-kit protein, a specific cell marker for stem cells. Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for c-kit and detected with a secondary antibody conjugated to Alexa Fluor 546 (red). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.

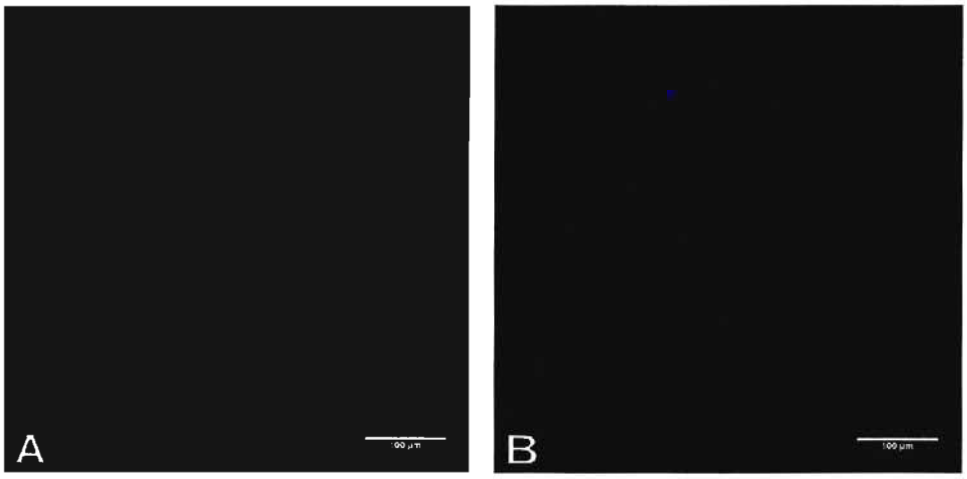

Figure A-5. Caspase-3 positive control. An aorta isolated from a normotensive rat (A) displays very little positive staining for caspase-3 (green). Similarly, an aorta isolated from a hypertensive rat (B) very little positive staining for caspase-3 (green). Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5μm. Samples were then immunostained for 3-nitrotyrosine and detected with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100μm.

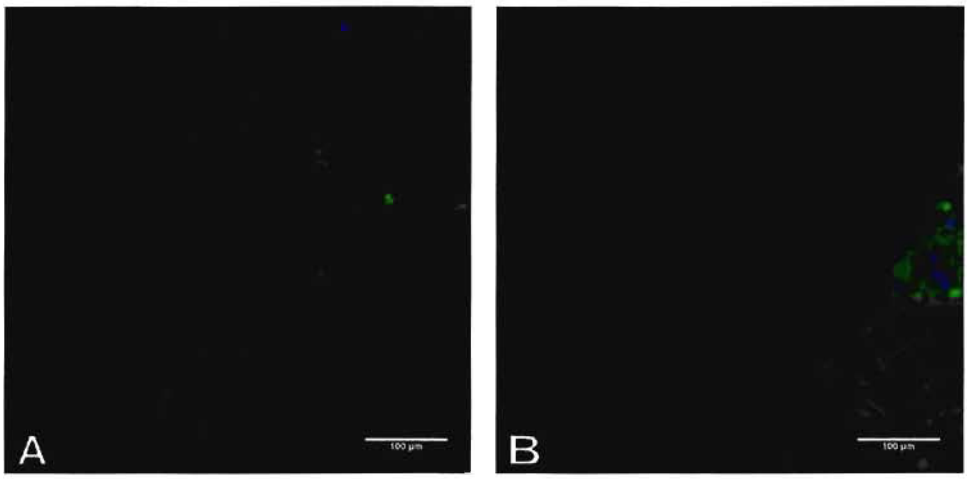

Figure A-6. 3-Nitrotyrosine positive control. An aorta isolated from a normotensive rat (A) displays very little positive staining for 3-nitrotyrosine (green). However, an aorta isolated from a hypertensive rat (B) displays significant positive staining for 3-nitrotyrosine (green). Thoracic aortas were isolated, formalin fixed, embedded in paraffin and serially sectioned at 5µm. Samples were then immunostained for 3-nitrotyrosine and detected with a secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were visualized using DAPI (blue). Samples were viewed under a Nikon fluorescent microscope with a 40X objective lens. Scale bars represent 100µm.